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# Estrogen Influences on the Developing Rat Hippocampus Through Interactions with the Brain Derived Neurotrophic Factor and P160 Coactivator Proteins

Derek Tyler Solum
*Loyola University Chicago*

LOYOLA UNIVERSITY CHICAGO

# ESTROGEN INFLUENCES ON THE DEVELOPING RAT HIPPOCAMPUS THROUGH INTERACTIONS WITH THE BRAIN DERIVED NEUROTROPHIC FACTOR AND p160 COACTIVATOR PROTEINS

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DEREK TYLER SOLUM

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LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| AP-1 | activator protein-1 |
| BDNF | brain-derived neurotrophic factor |
| CA1-3 | fields of the hippocampus (Cornu ammonis) according to Lorente de Nó |
| cDNA | complementary deoxyribonucleic acid |
| CNS | central nervous system |
| CREB | cAMP response element binding protein |
| DAB | diaminobenzadine |
| dATP | 3'-deoxyadenosine 5'-triphosphate |
| DHTP | dihydrotestosterone |
| E | estrogen |
| EDTA | ethylenediaminetetraacetic acid |
| EPSP | excitatory postsynaptic potential |
| ER ($\alpha$ and $\beta$) | estrogen receptor (subtypes $\alpha$ and $\beta$) |
| ERE | estrogen response element |
| ERKO | estrogen receptor knock-out mouse |
| GABA | $\gamma$-aminobutyric acid |
| GAD | glutamic acid decarboxylase |

| | |
|---|---|
| HBSS | Hank's buffered salt solution |
| ICC | immunocytochemistry |
| IPSP | inhibitory postsynaptic potential |
| ISH | *in situ* hybridization |
| LTD | long-term depression |
| LTP | long-term potentiation |
| mRNA | messenger ribonucleic acid |
| NGF | nerve growth factor |
| NGS | normal goat serum |
| NMDA | N-methyl-D-aspartate |
| OVX | ovariectomy |
| P | postnatal |
| PBS | phosphate buffered saline |
| RT-PCR | reverse transcription-polymerase chain reaction |
| SSC | saline-sodium citrate buffer |
| SDS | sodium dodecyl sulfate |
| T | testosterone |
| TAM | tamoxifen |
| Trk | tropomyosin-related kinase |

# ABSTRACT

In addition to their primary role in the maintenance and regulation of reproductive capacity, ovarian steroid hormones acting through specific nuclear receptors influence more general neurobiological functions, such as perceptual-spatial skills and learning and memory. Estrogens act to alter certain aspects of cognitive performance and pathology such as epileptic seizure activity and Alzheimer's disease. Furthermore, ovarian steroids act during development to alter the neuronal cytoarchitecture and synaptic connectivity. These early influences of steroids on the brain are essentially permanent and in part determine the steroid responsiveness of the adult brain. The mechanism and sites of action for the effects of estradiol on cognitive performance and epileptic seizure activity have not been established, but one probable site is the hippocampus, a steroid-responsive region of the brain. Estrogen receptors have been found in cells of the hippocampus using a variety of techniques. Furthermore, there is a transient increase in the expression of estrogen receptors during the first postnatal week of life; a period that is active in neuronal differentiation and synaptogenesis. During this time period estrogen has been shown to have a number of transcriptional effects, mediated by steroid receptor coregulatory proteins, on morphological and biochemical properties of hippocampal neurons that would predict an excitatory action of this steroid on synaptic physiology in the hippocampus.

Additionally, the neurotrophins, including brain-derived neurotrophic factor (BDNF), also affect the long-term survival and differentiation of hippocampal cells during development, and their viability in adulthood. Neurotrophins are widely expressed in the CNS and are involved in complex and activity-dependent modulation of dendritic and axonal growth. Moreover, the expression of neurotrophic factors has the ability to acutely modulate synaptic transmission by presynaptic as well as postsynaptic mechanisms. In addition to the modulation of synaptic transmission and plasticity, BDNF may also be involved in hippocampal synaptogenesis.

In the adult rat, estrogen regulates mRNA levels of BDNF and its high affinity receptor, trkB, in several regions of the rat brain. It follows, then, that estrogen induced changes in neurotrophin synthesis is an important mediator of differentiation of the brain, and specifically the hippocampus. Furthermore, hormonally mediated changes in neurotrophin expression during development may be causally related to plasticity in the adult hippocampus. The cellular mechanisms by which estrogen can influence the developing hippocampus have not been examined and is the focus of this outline. Information obtained will be contribute to a greater understanding of the hippocampus, and may illuminate some of the cellular and molecular events involved in the development, maintenance and decline of normal cognitive function.

# CHAPTER 1

# GENERAL INTRODUCTION

During development, estrogen has a variety of effects on the morphological, biochemical, and electrophysiological characteristics of neurons in several regions of the mammalian brain that result in sexually dimorphic brain structures in adulthood. The hippocampus is an estrogen responsive brain structure that is involved in cognitive functions such as spatial mapping, and learning and memory. In addition, it has been demonstrated that estrogen exerts a profound influence on the differentiation and plasticity of hippocampal neurons (Blanco et al., 1990; Gould et al., 1990). Gender differences in hippocampal morphology may arise due to the presence of testosterone that is aromatized to estrogen in nervous tissue by the enzyme aromatase (Naftolin et al., 1971).

The hormonal influences on the rat brain occur at an early period of development during late gestation and early postnatal life. This period has been defined as the "critical period" of sexual differentiation (Jacobson and Gorski, 1981). The early influences of steroids on the brain act to *organize* neural pathways, and these pathways are essentially permanent. Later in adulthood, steroids act on these differentiated neural pathways to *activate* behaviors and functions. This hypothesis was developed over forty years ago and is known as the organizational-activational hypothesis of steroid action (Phoenix et

al., 1959). It is during the critical period that the presence or absence of specific hormones has their impact upon brain development (McEwen, 1983). However, the mechanisms by which estrogen influences the developing brain, and specifically the hippocampus, is essentially unknown and is the focus of this dissertation project.

Estrogen acts by binding to intracellular estrogen receptors (ERs), of which there are two isoforms (alpha and beta; Greene et al., 1986; Tsai and O'Malley, 1994; Kuiper et al., 1996). Estrogen receptors act directly as transcription factors and are located predominantly in neurons. Moreover, both estrogen receptor isoforms (ERα and β) have been described in the neonatal hippocampus, implicating estrogen as an important influence in hippocampal development.

In addition to normal wild-type estrogen receptors, multiple mRNA splice variants of the estrogen receptor have been discovered in the normal rat brain, including the hippocampus (Petersen et al., 1998; Price et al., 2000). When transcribed *in vitro* these mRNA splice variants are functional proteins that exhibit altered ligand and DNA binding interactions and have a unique pattern of cellular localization. Thus, estrogen receptor mRNA splice variants add yet another facet to the possible molecular and cellular actions of estrogen.

Upon binding ligand, the ER complex interacts with an estrogen response element (ERE) in DNA where it regulates gene transcription and ultimately neuronal function. Estrogen receptors also interact with other proteins, termed nuclear receptor co-activators and co-repressors, that increase or decrease ER action at the ERE, respectively. In the absence of hormone, several nuclear receptors actively repress transcription of target

genes via interaction with nuclear receptor corepressors (NCoR). Upon binding of hormone, however, these corepressors dissociate from the DNA-bound receptor, which subsequently recruit a nuclear receptor coactivator (NCoA) complex. The coactivator proteins function as signaling intermediates between the receptors and the general transcriptional machinery to affect gene transcription (see Horwitz et al., 1996; Katzenellenbogen et al., 1996). Coactivator proteins accomplish this by interacting directly with the activation function (AF) domains of a nuclear receptor in an agonist dependent manner, leading to enhancement of receptor mediated transcriptional initiation. Over 30 potential coactivators have been identified by their ability to bind various receptor domains and to alter the transcriptional activity of NRs (McKenna et al., 1999a; Glass and Rosenfeld, 2000). Distinct classes of ligand-dependent transcriptional cofactors have been described, including CBP/p300 and the p160 family (Xu et al., 1999). At present, the p160 family contains three members including SRC-1/N-CoA1 (Onate et al., 1995; Kamei et al., 1996), SRC-2/GRIP-1/TIF2 (Hong et al., 1996; Voegel et al., 1996; Torchia et al., 1997) and SRC-3/AIB1/p/CIP (Torchia et al., 1997, Li, 1997, Suen, 1998). The temporal and spatial relationship of these regulatory proteins with the ERs could determine the specificity in the depression or activation of gene expression.

Interestingly, neurons in the adult rat forebrain of both sexes co-express estrogen and neurotrophins receptors and are the sites of estrogen action and neurotrophin synthesis (Miranda et al., 1993a). It is not surprising then, that in the adult hippocampus and hypothalamus, estrogen regulates several aspects of the neurotrophic system. For instance, estrogen has been shown to alter messenger RNA levels of brain-derived

neurotrophic factor (BDNF) and its high affinity receptor, trkB (Toran-Allerand, 1996; Gibbs, 1998). Brain-derived neurotrophic factor is a 14-kDa polypeptide that plays an important role in the survival, differentiation, and outgrowth of peripheral and central neurons during development and in adulthood (Barde, 1989; Davies, 1994). During postnatal life, BDNF has been shown to accelerate the development of both excitatory and inhibitory synapses in the hippocampus (Vicario-Abejon et al., 1998). BDNF exerts its effects on the nervous system via signaling primarily through trkB, and to a lesser extent through the low affinity pan neurotrophin receptor, $p75^{NGFR}$. The activated trkB receptors initiate local signaling at the membrane resulting in signaling cascades that traverse the cytoplasm and culminate with transcriptional changes (Segal and Greenberg, 1996). Recent studies indicate that within minutes of application, BDNF can influence synaptic transmission and plasticity in the hippocampus. It is possible then, that the trophic effects of estrogen on the hippocampus are caused by enhanced expression of neurotrophins and/or their receptors.

Considering these observations and data in the literature concerning estrogen's influence on the rat hippocampus, I hypothesized that during rat brain development, estrogen acts on the hippocampus by modulating brain derived neurotrophic factor, its receptor trkB, and/or the p160 coactivator proteins. The goal of this project was to describe the localization of estrogen receptors and coactivator proteins in the developing hippocampus, and further, to determine whether estrogen influenced the neurotrophic system early in hippocampal development. The information obtained from this dissertation may subsequently contribute to a greater understanding of estrogen's

organizational effects on brain structure and function. To achieve this goal, three specific studies were performed that utilized neuroanatomical, biochemical, molecular biological and *in vitro* cell culture techniques.

The purpose of Specific Aim 1 was to characterize the developmental expression of estrogen receptors (ER α and β) in the developing rat hippocampus. This aim employed Western blot analysis, receptor binding studies, immunocytochemistry and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) to determine the distribution and developmental expression of ERα and β in the rat hippocampus. Using immunocytochemistry, I analyzed the distribution of ERα in the developing male and female rat hippocampus at postnatal (P) days 0, 4, 7, 10, 15 and 20 and compared those ages with adults. The protein levels I observed *in vivo* were confirmed semi-quantitatively with Western blot analysis. Next, I used conventional and quantitative real-time PCR to analyze mRNA expression for ERβ splice variants in the developing rat hippocampus. Other brain regions, including the medial basal hypothalamus and cortex were also examined for comparison. Finally, I used an estrogen receptor binding assay to determine if the different ERs have distinct ligand binding affinities in the neonatal hippocampus. The data from this first specific aim are presented in Chapters 3 and 4 of this dissertation.

Based on data demonstrating effects of estrogen on the neurotrophic system in the adult, Specific Aim 2 was designed examine the hormonal regulation of BDNF and its receptor, trkB, in the CA1 and CA3 subregions of the rat hippocampus during early

postnatal development and is presented in chapter 5. This aim was accomplished by surgically removing endogenous hormones shortly after birth by gonadectomy. Immediately following gonadectomy, estrogen (or vehicle only) was replaced to mimic the early hormonal surge normally observed in intact animals. First, to determine whether estrogen receptors are colocalized with BDNF, or trkB, I used double-label immunocytochemistry on normal tissues and primary hippocampal cells. Initially, to determine if estrogen regulated BDNF mRNA levels *in vitro*, I treated hippocampal cells with multiple doses of 17β-estadiol benzoate, a long lasting estrogen receptor agonist. In these studies, BDNF mRNA levels were determined using quantitative real-time PCR. Subsequently, in the hippocampus and hypothalamus of hormone manipulated animals, mRNA transcript levels for BDNF and trkB were examined using quantitative real-time PCR. The last set of experiments for this aim examined whether estrogen alters protein levels for BDNF and trkB in the developing hippocampus using Western blot analysis.

To further elucidate the mechanisms by which estrogen influences the developing rat hippocampus, I designed a third series of experiments composing Specific Aim 3 to examine the expression of the p160 coactivators (including SRC 1-3) in the developing rat brain. These experiments were conducted to provide insight into the potential mechanisms by which estrogen receptors influence transcriptional activation during development. While the p160 coactivators likely play a significant role in gene transcription by steroid receptors, their expression in brain tissue during development had not been reported. Therefore, in the developing rat brain, I examined the mRNA expression for the p160 coactivators in the medial basal hypothalamus, hippocampus

regions CA1 and CA3, and the cortex using quantitative PCR. Additionally, I used immunocytochemistry to determine if the protein expression followed a pattern similar to the mRNA during development. Finally, I double-labeled hippocampal cells with ERα and SRC-1 to determine if these proteins we co-localized.

The overall conclusion from this dissertation project is that estrogen receptors (α and β) are present in hippocampal neurons and may mediate the effects of estrogen early in development by modulating brain derived neurotrophic factor. The effects of estrogen might also involve interactions with p160 family of steroid receptor coactivators. The influence of estrogen then, could have profound impacts on the developing hippocampus to organize various morphological, biochemical, and electrophysiological parameters of this brain structure.

# CHAPTER 2

# REVIEW OF RELATED LITERATURE

## *Structure of the hippocampus*

The hippocampus is a limbic system brain structure that plays an important role in cognitive functions including spatial mapping and learning and memory. As first shown by (Anderson and Lomo, 1966), the hippocampus is composed of a tri-synaptic excitatory circuit (Fig. 1). Accordingly, input identified as the perforant pathway is relayed from the entorhinal cortex to the granule cells in the hilus of the dentate gyrus. The axons of the granule cells form a bundle, termed the mossy fiber pathway, which in turn innervates the pyramidal cells of the CA3 region. Finally, the CA3 pyramidal cells send excitatory efferents, the Schaeffer collaterals, to the pyramidal cells of the CA1 region. The hippocampal formation therefore consists of a complex of three main subfields, the dentate gyrus, the CA3 subregion, and the CA1 subregion. The CA2 subfield is less well defined in the rat and appears to lack specific features and therefore is largely ignored in this outline. The granule cells of the dentate gyrus and the pyramidal cells of regions CA1-3 (Fig.2) are considered the principal cells of the hippocampus. These principal cells are excitatory, using the amino acid glutamate as a neurotransmitter.

Hippocampal outputs project to a variety of brain regions (Swanson et al., 1978) and act in a general way to integrate interoceptive and exteroceptive data

(Derryberry and Tucker, 1992). The output targets include the subiculum, entorhinal cortex, lateral septal nucleus, olfactory bulb, nucleus accumbens, amygdala, hypothalamus, and contralateral hippocampus.

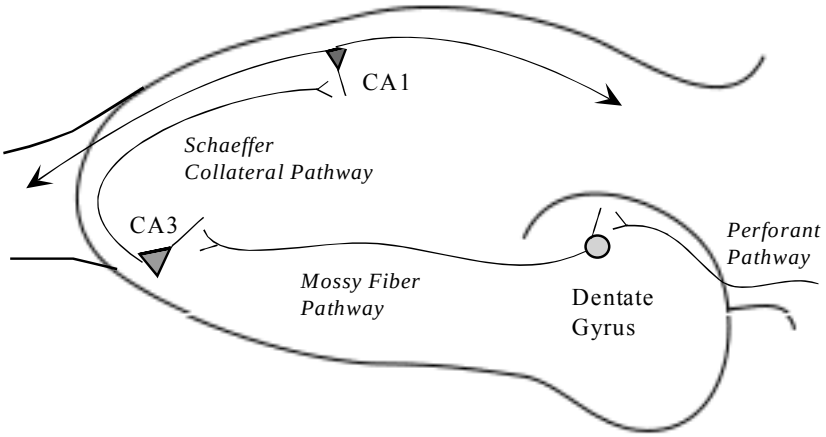



FIG 1: STRUCTURE OF THE HIPPOCAMPUS. This schematic diagram demonstrates the pattern of neuronal connectivity in the hippocampus. It is composed of a tri-synaptic excitatory network containing the dentate gyrus, and regions CA1-3.

There is some controversy concerning the inclusion of the dentate gyrus in the hippocampal formation. The dentate region has several morphologically distinct structures, including granule cells and the hilar polymorph region, which would support the view that this is a separate brain region altogether. However, the dentate gyrus sends strong efferents through the mossy fiber pathway to Ammon's horn, i.e., the CA subfields. Furthermore, lesion studies demonstrate that disruption of the dentate gyrus

results in the near abolishment of hippocampal function. Because of these anatomical and physiological connections, the dentate region is included with the hippocampus in this dissertation.

In contrast to the excitatory pyramidal and granule cells, the non-principal hippocampal local circuit neurons synapse locally and project to the contralateral hippocampus and subcortical structures. These are considered to be inhibitory interneurons, which synapse primarily on the soma and proximal dendrites of pyramidal cells (Ribak et al., 1978). Most inhibitory interneurons (~97%) in the brain use the amino acid γ-amino butyric acid (GABA) as a neurotransmitter and are responsible for modulating excitatory responses of pyramidal cells in the adult brain. Hippocampal pyramidal cells, in contrast, excite postsynaptic neurons by liberating an excitatory amino acid. Hippocampal inhibitory cells are diverse and are thought to fall into functionally distinct subsets defined by a similar morphology and physiology. Those inhibitory cells that innervate pyramidal cell somata or axon initial segments control the local generation of $Na^+$-dependent action potentials and inhibitory fibers terminating on dendrites can suppress calcium-dependent spikes (Miles et al., 1996). Thus, distinct inhibitory cells may differentially control dendritic electrogenesis and axonal output of hippocampal pyramidal cells. In addition, the discovery that some inhibitory interneurons form synapses that contact only other inhibitory cells (Gulyas et al., 1996) implies that local inhibitory cell networks also exist. Hippocampal interneurons contain a variety of neuro-peptides, which may act as co-transmitters, as well as several calcium binding proteins (Freund and Buzaki, 1996). Calcium-binding proteins, including parvalbumin, calbindin

28k, and calretinin, act to buffer the influx of calcium as a result of excitatory stimulation and activation of L-type $Ca^{2+}$ channels and can be used to differentiate the subsets of hippocampal interneurons.

## *Principal cells of the hippocampus*

Pyramidal cells are the excitatory projection neurons of the hippocampus and use L-glutamate as a transmitter. In the major regions of the hippocampus (CA1-3), the pyramidal cells are situated in a single continuous layer called the stratum pyramidale. In contrast to other neuronal types, pyramidal cells have not one, but two dendritic trees which emerge from opposite sides of the cell body (Fig. 2). The basal dendrites arise from the side that gives rise to the axon, and the apical dendrites arise from the other side.

Excitatory input to CA1 pyramidal cells is extensive. About 5000 CA3 pyramidal cell axons from the Schaffer collateral pathway converge to synapse on a single CA1 cell (Kandel et al., 1991). These synapses form at all levels of the CA1's dendritic tree, from close to the cell body to more distant levels. Most of the synapses are excitatory upon dendritic spines in area CA1. Because the neck of the dendritic spine restricts diffusion between the head of the spine and the rest of the dendrite each spine may function as a separate biochemical region (Harris and Stevens, 1989). This compartmentalization has important implications for selectively altering the strength of synaptic connections during learning and memory.

Extensive morphological studies have demonstrated that even within the hippocampus there is some variation in the extent of pyramidal cell dendritic branching.

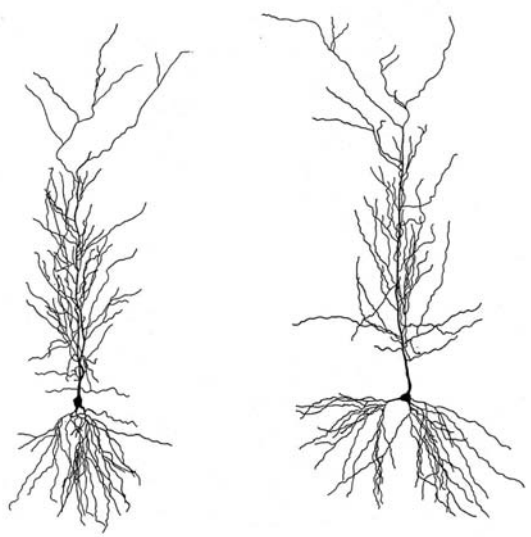


FIG 2: PRINCIPAL CELLS OF THE HIPPOCAMPUS. Camera Lucida drawings of biocytin-filled CA1 pyramidal cells demonstrate the elaborate apical and basal dendritic arborizations. Adapted from Cannon (1998).

These studies indicate that dentate granule cells have dendritic trees that averaged 9,300μm in total length. In contrast, CA1 and CA3 pyramidal cells have much more elaborate dendritic trees, averaging 13,500 and 15,800μm in total length respectively (Ishizuka et al., 1995). However, many more characteristics may be used to identify either similarities or differences between the classes of hippocampal pyramidal cells. For example, van Pelt et al., (1997) hypothesized that the differences between pyramidal

neurons is that branching probabilities may decrease with distance from the soma more rapidly in the simpler cells than in more complex cells.

During late embryonic and early postnatal development, hippocampal pyramidal cells are undergoing synaptogenesis. It is at this time period that the structure of neurons and their circuits are formed. Subsequently, the local environment plays a critical role in the development and maturation of the neuronal circuitry of this system. For instance, pyramidal, but not non-pyramidal, dendrites are influenced by chronic exposure to neurotrophic factors during the neonatal period (Baker et al., 1998).

### *Non-Principal cells of the hippocampus*

The pioneering Golgi stain studies of Ramon y Cajal (Cajal, 1893, 1911) and Lorente de Nó (1934) have firmly established the notion of several heterogeneous classes of non-principal neurons, generally referred to as interneurons. In the hippocampus, interneurons are considerably less abundant (<10%) than spiny principal neurons, such as granule and mossy cells of the dentate gyrus and pyramidal cells of regions CA1-3 (Olbrich and Braak, 1985). Moreover, following recent technical and conceptual progress, an astonishing variety of interneuron classes have been identified (Freund and Buzaki, 1996).

It has been established that there are at least 16 distinct morphological interneuron phenotypes and 3 different modes of discharge (Parra et al., 1998) in the hippocampus. Axons of different groups of inhibitory cells target precise and different zones of

pyramidal cell membrane (Fig. 2; Buhl et al., 1994; Miles et al., 1996). These inhibitory cells predominantly use the fast acting amino acid GABA as their

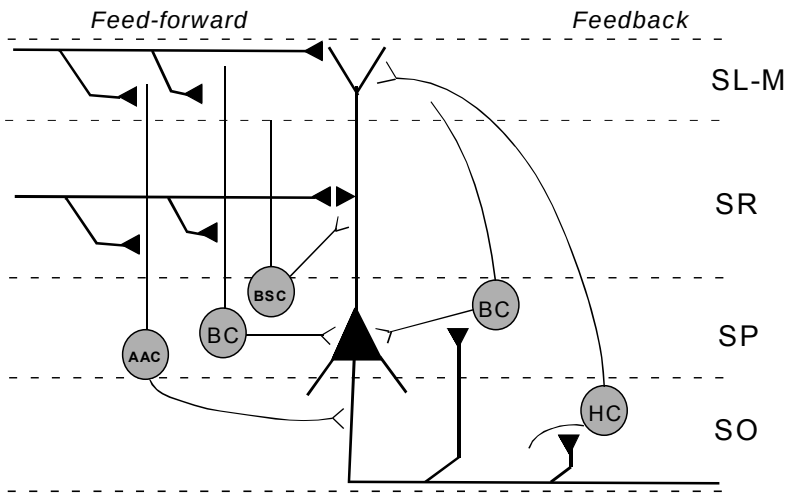



FIGURE 3: GABAERGIC CELLS IN THE HIPPOCAMPUS. Principal cells (black) are contacted by GABAergic interneurons (gray) via feed-forward (left) and feedback (right) circuits. Interneurons can be divided into distinct classes according to the input they receive and their output profiles (as defined by the selective targeting of membrane domains of principal cells). AAC, axo-axonic cells; BC, basket cells; BSC, bi-stratified cells; HC, horizontal cells; SL-M, stratum lacunosum-moleculare; SR, stratum radiatum; SP, stratum pyramidale, SO, stratum oriens.

neurotransmitter. Although interneurons have been viewed traditionally as subserving an inhibitory function, recent anatomical studies indicate that those interneurons that selectively innervate other interneurons may have a net disinhibitory activity without direct effect on principal cells (Freund and Buzaki, 1996). Moreover, GABAergic inhibitory post-synaptic potentials (IPSPs) are depolarizing during early development

(Cherubini et al., 1991) and are instrumental in synchronizing population firing rates (Cobb et al., 1995). The principal cells of the hippocampus are contacted by GABAergic interneurons via feed-forward as well as feed back circuits (Fig. 3). The three main types of GABAergic interneurons are the axo-axonic, basket, and bi-stratified cells. These interneurons can be divided into distinct classes according to the input they receive, as reflected by the layer-specific location of their dendrites; and their output profiles, as defined by the selective targeting of membrane regions of principal cells. Basket cells provide somatic and perisomatic inhibition (Buhl et al., 1994) whereas other types of interneurons synapse primarily onto subdomains of the dendritic tree (Freund and Buzaki, 1996; Miles et al., 1996). It thus appears that interneurons are a very diverse population acting in a variety of ways to influence the neurotransmission of cells in the hippocampus.

### *Function of the hippocampus*

The hippocampus projects to several brain regions and acts to facilitate cognitive functions such as spatial mapping and learning and memory. Storage of information as long-term memory is commonly assumed to involve modifications of the relevant synapses. Studies have demonstrated that a brief high-frequency train of stimuli to any one of the three afferent pathways of the hippocampus produces an increase in the excitatory synaptic potential in the postsynaptic hippocampal neurons. This effect, which can last for hours, days and even weeks (Bliss and Lomo, 1973), has been termed long term potentiation (LTP). LTP, a use dependent increase in synaptic efficacy, is

considered the major experimental model for learning and memory formation at the synaptic level in mammals and, in the extreme, epileptic activity. Studies have shown that LTPs at different synapses in the hippocampus are not identical. In the CA1 region, LTP has three interesting properties: 1) cooperativity (more than one fiber must be activated to obtain LTP), 2) associativity (the contributing fibers and the postsynaptic cell need to be active together, in an associative way), and 3) specificity (LTP is specific to the active pathway) (Kandel et al., 1991). Using spatial and visual water maze tasks, Morris and colleagues (1986) have demonstrated that the associative LTP in CA1 is important for spatial memory. The CA3 region, in contrast, has different properties and is not associative. Additionally, LTP in the CA3 does not require an increase in intracellular calcium levels. In fact, the CA3 region may not be involved in LTP at all.

Normal hippocampal function in the adult is dependent on precise interactions between the excitatory glutamatergic pyramidal neurons and inhibitory GABAergic neuronal systems. Pyramidal neuron firing rates reflect not just excitatory drive but the balance between excitatory inputs received from other pyramidal neurons and inhibitory inputs received from GABAergic interneurons. Supporting the role of the hippocampus in learning and memory, administration of GABA agonists muscimol and baclofen impair these functions, while antagonists facilitate memory retention in the adult rat (Ammassari-Teule et al., 1991). Excess stimulation (Feldblum et al., 1990) or inhibition (Houser, 1991) of GABA activation can result in epileptiform activity. The hippocampus is particularly susceptible to the ischemic damage and trauma that has been associated with increases in epileptiform activity and seizures.

Electrophysiological studies of hippocampal "place" cells (O'Keefe and Conway, 1978) and hippocampal long-term potentiation support hypotheses of the important role of the hippocampus in spatial memory processing. Furthermore, neurobehavioral studies consistently show that hippocampal damage is associated with disruption of spatial learning and memory processing for a variety of adult species including humans (Olton and Papas, 1979).

## *Development of the hippocampus*

The time course of hippocampal development is extensive. It begins prenatally and continues until well after birth. Examination of the postnatal development of pyramidal neurons in rats shows extensive growth of the dendritic tree. However, not all components develop simultaneously. The basal dendrites and the shaft and terminal branches of the apical dendrites develop first (before postnatal day 15). These immature neurons have a small soma and only short, simple, often bipolar processes (Cowan et al., 1980). During the next few days, the apical dendrites elongate into the molecular layer as the lateral and pre-terminal branches of the apical dendrites proliferate. These cells begin forming synapses with the axons of cells in the tri-synaptic network, and as a result dendritic spines increase in density and change shape to reach maximal numbers by postnatal day 24 (Pokorny and Trojan, 1986).

The hippocampus is similar to the neocortex in that the GABAergic interneurons are generated embryonically, between embryonic days 14 and 18. These interneurons become postmitotic before the pyramidal and granule cells (Amaral and Kurtz, 1985;

Lubbers et al., 1985). The interneuron's circuitry is well developed and morphologically mature by postnatal day 7 (Seay-Lowe and Claiborne, 1992) even as their target granule and pyramidal cells excitatory connections are still being developed. Thus, the GABAergic system is well developed at birth (Coyle and Enna, 1976; Rozenberg et al., 1989). In contrast, the excitatory inputs begin developing after the first postnatal week (Richter and Wolf, 1990). The principle and most numerous cells of the hippocampus are the granule cells of the dentate gyrus and the pyramidal cells found in regions CA1 to CA3. Granule cells are considerably smaller than pyramidal cells, and are subsequently more numerous as well. Pyramidal cells begin development prenatally, in contrast to cells of the dentate gyrus where 80% of cells are generated postnatally (Bayer, 1980). In the subgranular zone of the dentate gyrus, neurogenesis continues for at least one year (Schlessinger et al., 1975), and likely throughout the life of the organism (Gage et al., 1998). This process of neurogenesis, which includes proliferation, survival, migration and differentiation, can be regulated by a variety of stimuli. It appears that the total granule cell number increases into midlife of rats and mice and then reaches a plateau, but does not decline (Kempermann et al., 1998). Furthermore, there is little postnatal neuronal death occurring in the hippocampus of the rat (Boss et al., 1987).

Fewer studies have examined the effects of hippocampal damage sustained during infancy on the development of spatial learning and memory abilities. However, it has been shown that neonatal hippocampal damage can result in long-lasting if not permanent deficits in spatial memory (Altemus and Almli, 1997). These results showed that behavior performance measures under the spatial conditions were significantly correlated

with the total volume of hippocampal tissue damage. Accordingly, hippocampal integrity is important for the normal development of spatial learning and memory functions, and other brain structures do not assume hippocampal-spatial memory functions when the hippocampus is damaged during the neonatal period. It is to be stated, above all, that while the conditions for neonatal learning have not been elucidated, newborn animals are able to learn and memorize a number of reactions, even several hours after delivery and remember them for 24 hours (see Myslivecek, 1997).

### *Mechanisms of sexual differentiation*

Testosterone (T) is not only a hormone but a prohormone, the precursor of estradiol, resulting from aromatization, and of 5α-dihydrotestosterone (DHT) via the 5α-reductase system. The enzyme aromatase was described in neuroendocrine tissues more than 25 years ago (Naftolin et al., 1971), which created the foundation for the "brain aromatization hypothesis" (Naftolin and Ryan, 1975). Subsequent experiments demonstrated that treatment with DHT, the non-aromatizable androgen, was ineffective in increasing masculinization of females (McDonald and Doughty, 1972; Gerall et al., 1976) or restoring normal male mating behavior (McDonald et al., 1970; Baum et al., 1982). The studies implying that regulation of male sexual behavior required the conversion of androgen to estrogens, presumably through the aromatization, are reviewed by Lephart (1996). Both T and DHT bind with high affinity to androgen receptor (AR) whereas 17β-estradiol will preferentially bind the estrogen receptor. The

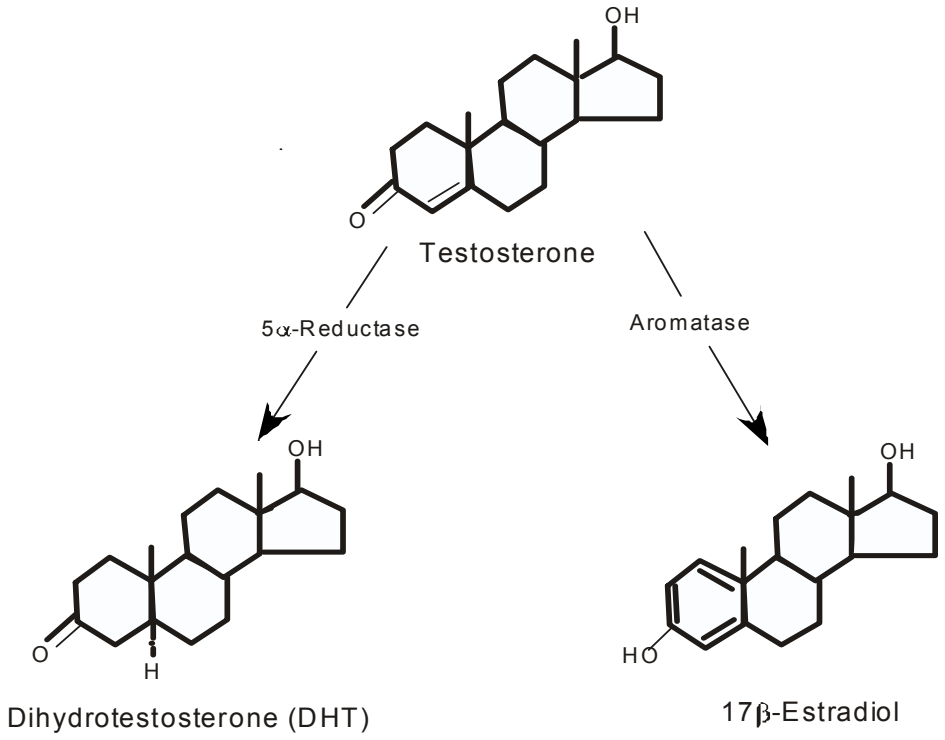



Fig 4: BIOCHEMICAL PATHWAY FOR THE METABOLISM OF TESTOSTERONE. This diagram illustrates the conversion of testosterone to 17β-estradiol via aromatization, and Dihydrotestosterone (DHT) via the p450 enzyme 5α-reductase.

findings of these early rat studies supported the hypothesis that many, if not most, of the effects of androgens on neural cell differentiation are mediated by the local formation of estrogens by the aromatase enzyme.

*The adult hippocampus shows a response to estrogen*

The hippocampus is one site in the brain that shows a robust responsive to estrogen, and ER transcripts have been localized there as well (Register et al., 1998). The hippocampus is not only involved in learning and memory functions, but has been

implicated in some reproductive characteristics like female sexual behavior as well (Cameron et al., 1979). In the adult, sex differences have been observed in the CA1 and CA3 region where males and females differ in neuronal branching patterns and dendritic spine densities (Gould et al., 1990) and synapses (Woolley and McEwen, 1992). When ovariectomized rats are treated with estradiol, an increase in spine density occurs on the apical dendrites of the pyramidal cell in the CA1 region (Gould et al., 1990; Woolley and McEwen, 1993). It has been suggested that these effects on pyramidal cells are mediated through a mechanism dependent on the activation of N-methyl-D-aspartate (NMDA) receptors (Woolley and McEwen, 1994). These results are further supported by experiments demonstrating that estradiol regulates mRNA and protein of the NMDAR1 subunit in the hippocampus of adult ovariectomized female rats (Gazzaley et al., 1996). In addition, it has been shown that following estrogen treatment, the amplitude of both sustained and transient $Ca^{2+}$ currents, but not $K^{+}$ currents, are significantly enhanced when compared to non-treated OVX controls (Joels and Karst, 1995). Consistent with the data of Woolley et al. (1997), the alteration of $Ca^{2+}$ by the long-term modulation of estrogen could be linked to steroid-induced morphological changes in dendrites of CA1 pyramidal cells.

The dentate gyrus is also different between the sexes. Pfaff (1966) originally reported a greater cross sectional area in the dentate gyrus of adult male rats, and it is heavier in adult male than female rats (Madeira and Paula-Barbosa, 1993). In this region, males have a thicker granule cell layer (Juraska et al., 1988) and greater granule cell size, number and density in comparison to females (Wimer and Wimer, 1989). Moreover,

dendritic branching patterns of dentate granule cells have been reported to be different between the sexes (Juraska et al., 1985), and more mossy fiber synapses are found in males than females (Parducz and Garcia-Segura, 1993). These phenotypic sex differences can be altered in females by postnatal exposure to testosterone (Roof and Havens, 1992).

Inquiries into the functional consequences of these changes using intracellular recording techniques have showed that estradiol administration increases synaptic excitability and prolonged the EPSP in 20% of pyramidal neurons in the CA1 region (Wong and Moss, 1992). This is consistent with the pioneering work by Terasawa and Timiras, (1968) who showed that estradiol treatment decreases hippocampal seizure threshold. This work has been extended to show that induction of long term potentiation is maximal in female rats during the afternoon of proestrus when estrogen levels are high (Warren et al., 1995; Cordoba Montoya and Carrer, 1997). These differences in the hippocampus have also been demonstrated in different aspects of behavior. Accumulating evidence from a variety of experimental human (Phillips and Sherwin, 1992; Kampen and Sherwin, 1994; Kimura, 1995) and animal (Luine and Rodriguez, 1994; Diaz-Veliz et al., 1995; Kampen and Sherwin, 1996; O'Neil et al., 1996; Packard et al., 1996) data indicate that estradiol may affect learning and memory processes.

### *Sexual differentiation of the brain*

Individual differences in brain function and behavior are shaped partly by the effects of early experience and by hormones. Sexual differentiation of the brain is an

example of a hormonally directed event in which the presence or absence of testosterone during fetal and neonatal life causes brain development to diverge in two directions: male or female. During normal development, the brain is exposed to high

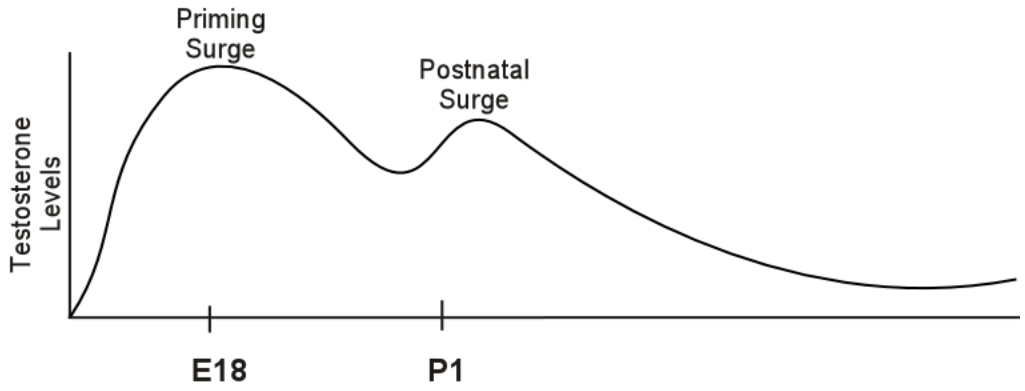



FIG 5: TESTOSTERONE SURGES DURING LATE EMBRYONIC AND EARLY POSTNATAL DEVELOPMENT IN THE MALE RAT. In the male rat, a priming surge of testosterone is observed beginning on embryonic (E) day 17, and peaking at E18-19. AT E18 the levels of testosterone peak, and are significantly greater than females. A second surge of testosterone is observed at birth, which peak 2 hours after delivery.

levels of gonadal steroids during a perinatal sensitive period. In rodents, males are exposed to higher testosterone levels than females during the perinatal period (Weisz and Ward, 1980; Rhoda et al., 1984). In males, the testes of the embryo begin testosterone secretion with a priming surge on embryonic day 18 and at this point the levels are significantly greater than observed in females (Weisz and Ward, 1980). Immediately following birth, a second smaller testosterone surge is observed in males (Fig. 5; Hoepfner and Ward, 1988). This period has been defined as the "critical period" of sexual differentiation beginning in late gestation and ending in early neonatal life. The

sensitive period for sexual differentiation of the rat hypothalamus is approximately E18 to postnatal days 7-10 (Jacobson and Gorski, 1981). Evidence for a critical period in hypothalamic development comes from studies showing that steroidal effects are not seen after about the tenth postnatal day in rats (Lobl and Gorski, 1974). It is during this period that the presence or absence of specific hormones has their impact on development. Testosterone exerts an organizational influence on a plastic CNS during this period (McEwen, 1983).

It has generally been considered that the default pattern of the nervous system is female (MacLusky and Naftolin, 1981), resulting from a lack of hormonal stimulation. However, these suggestions were challenged by studies showing that endogenous serum e subsequent studies (Nunez et al., 1971) demonstrestrogen levels are extremely high during the postnatal period in both male and female rats (Weisz and Gunsalus, 1973; Ojeda et al., 1975). Whilated that the estrogen binding protein, alpha feto-protein, binds estrogen and prevents its entry into the brain, secretions from the ovaries may also be important. For instance, because it acts as a transcription factor when bound to its receptor, very little estrogen may be needed to have profound effects on neurons. Alternatively, rather than preventing estrogen from entering the brain, α-feto-protein may act as a carrier, delivering estrogen to hormonally responsive tissues. Additional studies showing that estrogen, secreted from the ovaries, has important developmental consequences support this view. For instance, Gerall et al., (1972) and Hendricks and Duffy, (1974) demonstrated that the presence of an ovary transplanted into neonatally castrated male rats actively feminized their behavior. This effect is mimicked by

estradiol benzoate treatment from 20 to 30 days after birth, given to males castrated on day 1 (Hendricks and Gerall, 1970), suggesting that the effects of neonatal estrogen may last longer than the first postnatal week. Even though the time frame for these studies is quite variable, they underscore the importance of estrogen's actions during development.

The early influences of steroids on the brain act to *organize* neural pathways responsible for reproductive behavior, and these pathways are essentially permanent. Later in adulthood steroids act on these differentiated neural pathways to *activate* behaviors and functions. They in part determine the steroid responsiveness of the adult brain. This paradigm is known as the organizational-activational hypothesis of steroid action and was developed by Phoenix et al., (1959), who along with Barraclough and Gorski, (1961) and Harris and Levine, (1962) were the first to propose that gonadal steroids acted on the brain.

Furthermore, neurons of the developing brain have high levels of the P450 enzyme, aromatase, which converts significant amounts of gonadally derived testosterone to estrogen in the brain. Aromatase mRNA levels increase dramatically before birth and peak on postnatal days 3-4, after which they decline to adult levels (Harada and Yamada, 1992). Transiently high increases of aromatase have also been described in the developing rat hippocampus (MacLusky et al., 1994). The aromatization of testosterone is a necessary event in the "masculinization" of the rodent brain, and estrogen acting through its receptor is the masculinizing hormone (Naftolin and Ryan, 1975). In the absence of testis or the androgen surge during late gestation, the brain remains "feminized" (Arnold and Gorski, 1984). Furthermore, testosterone facilitation of male

sexual behavior can be blocked by a steroidal inhibitor of aromatization (Goy and McEwen, 1980). So, in the rodent brain, it is the levels of estrogen that control masculinization.

Steroidal modulation in the brain is important for a number of neurological functions. Many of these functions are not directly associated with reproductive function. For instance, levels of circulating estrogen influence neuroendocrine regulation, sexual differentiation of the brain, mood, learning and memory, responses to stress, body temperature, running activity, food ingestion, aggression and sensory sensitivity (McEwen, 1994). While many components of this process are well established, the cellular mechanisms mediating hormonally induced sexual differentiation of the brain remain largely unknown.

### *Evidence for estrogen's action on the neonatal hippocampus*

As described above, estrogen has been shown to induce synapse formation in hippocampal CA1 neurons in adult rats, which is thought to be determined by the neonatal actions of testosterone during development. Estrogen treatment of adult males fails to produce the same increase in synaptogenesis as in females. If the aromatization of testosterone to estradiol is blocked during development, however, males demonstrate a female-like response to estradiol treatment in adulthood with similar increases in spine synapses (Lewis et al., 1995). In a recent study examining the hippocampal volume and neuron number of food caching squirrels it was determined that seasonal variation of sex differences in the morphology of the hippocampal complex previously reported in short-

lived rodents might not reflect true adult plasticity, but rather results from organizational or activational effects of gonadal hormones during development (Lavenex et al., 2000). This study used modern stereological techniques to determine that seasonal changes in behavior associated with increases spatial memory processing do not correlate with morphological changes, such as variations in volume or neuron number.

Organizational effects on hippocampal mediated behaviors are suggested by the ability of castration in neonatal male rats to produce female learning patterns in adults, and estradiol treatment of neonatal females to produce male-typical learning patterns (Williams and Meck, 1991). Additionally, it has been shown that males perform better than females using spatial cues (Williams et al., 1990; Williams and Meck, 1991), generally considered a hippocampus mediated behavior, and these differences can arise as a result of the neonatal exposure of males to endogenous testosterone (Williams et al., 1990; Williams and Meck, 1991; Roof and Havens, 1992). More recent data in mice lacking ERα suggest that this receptor may mediate the effects of steroid hormones in a spatial learning task (Fugger et al., 1998). Similarly, humans females demonstrated significantly enhanced spatial ability when exposed to excessive androgens during development as compared to normal male relatives (Nass and Baker, 1991).

### *The Estrogen Receptor*

Many of the actions of estrogen are exerted via the estrogen receptor (ER). The ER is an intracellular receptor that is a member of a large nuclear receptor superfamily of proteins. Estrogen receptors function directly as ligand-dependent transcription factors

regulating the synthesis of specific RNAs and proteins. These receptors have a highly conserved DNA-binding domain and a less conserved C-terminal ligand-binding domain (Evans, 1988). The primary role of ER is to regulate the expression of specific target genes by binding DNA directly through estrogen response elements (ERE), often located in the 5' flanking region of estrogen responsive genes. The ER may also affect transcription indirectly through interactions with transcription factors such as the activator protein 1 (AP-1). The actions of estrogens are antagonized by antiestrogens, which bind to the ER in a manner that is competitive with estrogen; but antiestrogens usually fail to effectively activate gene transcription (Katzenellenbogen, 1996). The estrogen receptors are similar to most other steroid hormone receptors (Fig. 6), in that

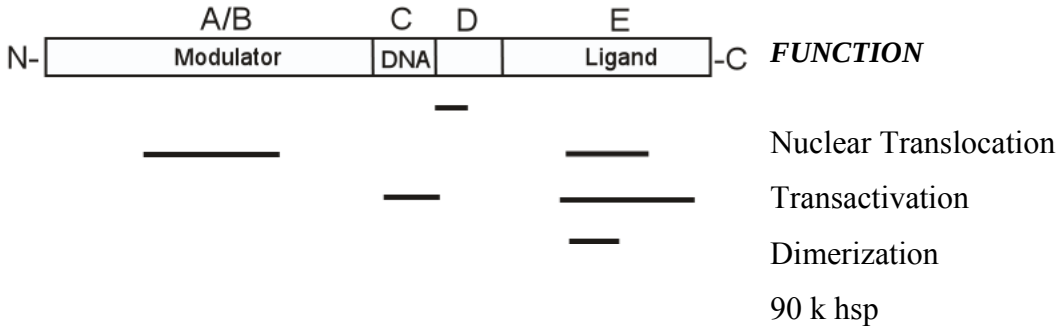



FIG 6: GENERAL STRUCTURE AND FUNCTIONAL ORGANIZATION OF NUCLEAR RECEPTORS-This diagram represents a linearized steroid hormone receptor and its domains. The modulator domain (A/B) mediates protein-protein interactions with other transcription factors. The DNA binding domain (C) is highly homologous across members of the steroid family and contains two zinc finger motifs. The 15mer DNA consensus sequence for ER binding is AGGTCAnnnTGACCT. The ligand binding domain (E) is located at the C-terminus and contains amino acid residues necessary for hormone binding.

they exhibit a variable N-terminal region, a short and well-conserved cysteine-rich DNA binding central domain, and a relatively well-conserved ligand binding C-terminal half (Kumar et al., 1987). The carboxyl terminal domain is both structurally and functionally complex. In addition to its hormone binding and nuclear translocation roles, the ER contains *trans*-activation and dimerization functions.

The central domain contains a cysteine rich region compatible with the formation of DNA binding zinc fingers (Miller et al., 1985). This domain of 66-68 amino acids binds to a specific region of DNA sequence termed the hormone response element (HRE). These response elements are short, palindromic sequences of DNA that enable the hormone receptor to bind with high affinity (Klein-Hitpass et al., 1988). The N-terminal domain is less well defined and may have a modulatory effect on *trans*-activation. It may also provide gene specificity for transcriptional activation by mediating protein-protein interactions with other general transcription factors. Although the precise mechanism by which ER regulates transcription remains to be determined, considerable progress has been made in defining the domains within ERα required for its activity. Specifically, it has been demonstrated that the transcriptional activity of ERα is mediated by two activation functions (AFs) located in the amino-terminal (AF-1) and carboxyl-terminal (AF-2) regions. AF-1 in the N-terminal domain, whose activity is regulated by phosphorylation in response to growth factors (Kato et al., 1995; Bunone et al., 1996), and AF-2 in the hormone-binding domain (HBD), whose activity is strictly dependent on the presence of ligand. Although both of these AFs function in a synergistic manner in most circumstances, they can also function independently in a cell-

and promotor-specific manner, an activity that may explain the tissue-selective agonist activity of some ER ligands (Berry et al., 1990; Tzukerman et al., 1994). The activity of estrogen receptor function is highly dependent on its conformation. The ER can exist in ligand bound and unbound states, and are predominantly located in the cell nucleus, irrespective of whether they are bound to estradiol (Greene et al., 1984). Furthermore, it has been demonstrated that different heat shock proteins, including hsp70 and hsp90, can bind to the ER, and after doing so, maintain the receptor in a receptive but transcriptionally inactive state (Pratt, 1990). The heat shock proteins likely assist in the folding of the receptor protein and/or transport across membranes (Smith and Toft, 1993) in addition to maintaining the receptor's structure. Upon hormone binding, the hsp90 protein dissociates and the ER dimerizes with itself. Following dimerization, the estrogen receptor binds with high affinity to DNA at the estrogen response element (ERE) and transcription is initiated (Fig 7).

Previous studies have shown that estrogen treatment of neonatal rats can cause the transformation of the hippocampal ERs from the cytoplasmic to the nuclear form and that bound ER can bind DNA (O'Keefe and Handa, 1990). In the juvenile (Orikasa et al., 2000) and adult (Weiland et al., 1997) hippocampus the ER's are found in the greatest concentration predominantly in interneurons in the hilar polymorph region of the dentate as well as in interneurons of the radiatum of CA3. In contrast, in the neonate, ER mRNA (O'Keefe et al., 1995) and protein (Solum and Handa, 2001) is highly concentrated throughout the pyramidal cell layer of CA3, and to a lesser extent in CA1, demonstrating a presence in pyramidal cells and interneurons.

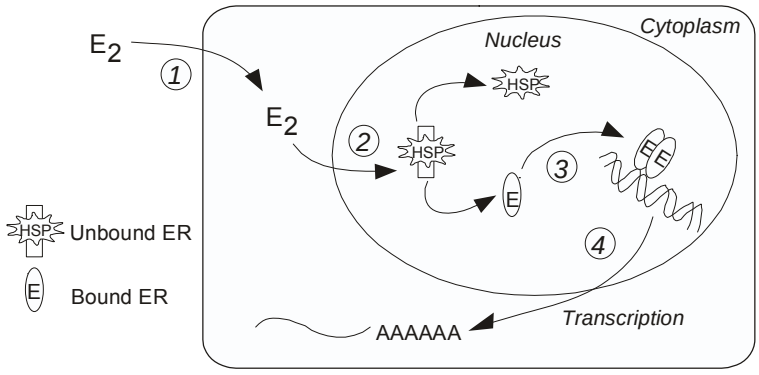



FIG 7: A SCHEMATIC DIAGRAM DEMONSTRATING THE CLASSICAL ESTROGEN RECEPTOR ACTIVATION PATHWAY 1) The steroid hormone passes through the cell membrane and 2) binds to the receptor. Binding of E to its receptor displaces the hsp and transforms the receptor. 3) The transformed receptor dimerizes and binds DNA at the HRE to 4) initiate transcription of estrogen responsive genes.

*Non-classical actions of estrogen*

Although modification of gene expression as a consequence of estrogen liganding to DNA-binding receptors is the traditional framework for interpreting underling mechanisms, an increasing number of reports document effects of acute application of estrogenic steroids that are too rapid (occurring within 10 minutes) to be accounted for by a genomic pathway. A novel pathway for the action of estrogen on the nervous system has been demonstrated by studies producing rapid (2-3 minute) and reversible changes in membrane excitability after steroid application (Fig. 8; Moss et al., 1997). Effects of estrogen on the electrophysiological activity of rodent hippocampal neurons was first

reported by (Teyler et al., 1980), who found that 17β-estradiol treatment induced a rapid enhancement of glutamatergic synaptic transmission in the CA1 region of in vitro hippocampal slices. While little is known about the molecular identity of the binding site for the rapid action, there is evidence that some rapid action may involve membrane or intracellular receptors that are coupled to ion channels and transmitter receptors by second messengers (Mermelstein et al., 1996). Early studies have shown that estrogen can alter cyclic nucleotide levels in the cell (Guanaga et al., 1974; Weissman et al., 1975), and act on potassium channels via a second messenger system involving cAMP to

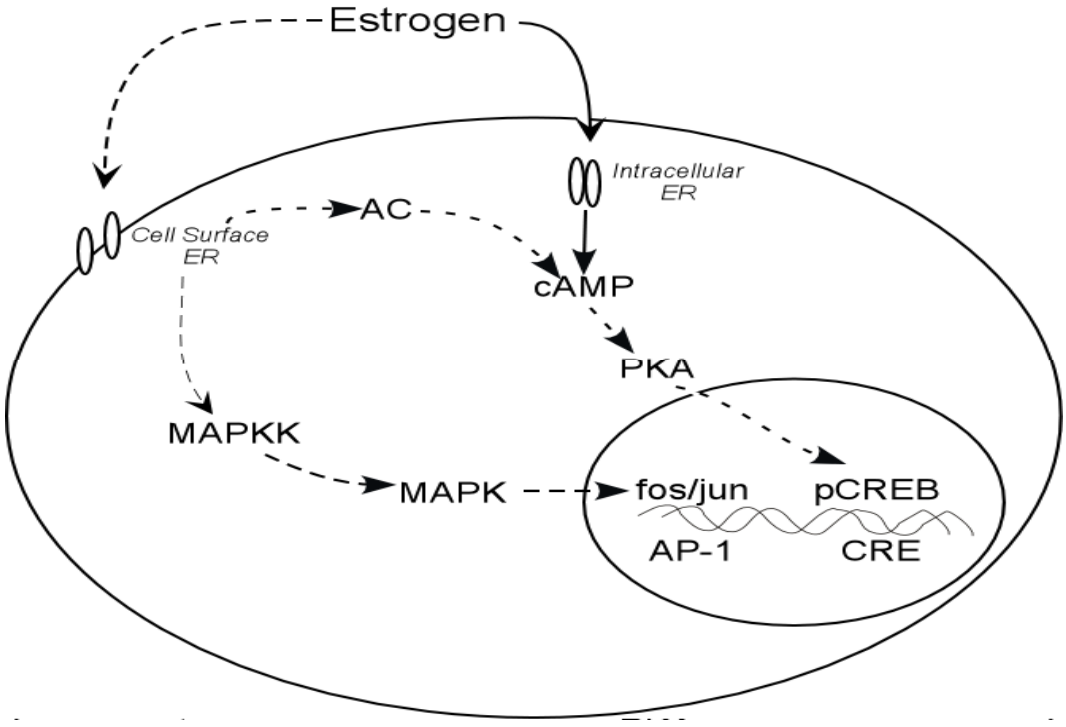



FIG 8: NONGENOMIC EFFECTS OF ESTROGEN. Possible nongenomic effects mediated by estrogen involve the MAP Kinase and cAMP cascades. These effects might be mediated by a classical intracellular ER (solid arrows) or via a novel membrane receptor (dashed arrows). Following signal transduction, gene transcription is actived through an CRE or AP-1 response elements. AC, Adenylate cyclase; pCREB, phosphorylated cAMP response element binding protein; MAPK, MAP kinase; CRE, CREB response element; PKA, protein kinase A.

modulate the membrane potential (Minami et al., 1990). It appears then, that estrogen may also act through non-transcriptional mechanisms to function in a neuromodulatory role. For instance, estrogen treatment of ovariectomized rats rapidly increases immunoreactivity for the phosphorylated form of the cAMP response element binding protein (CREB). This has been demonstrated in neurons of the preoptic area, the bed nucleus of the stria terminalis (Zhou et al., 1996) and the anteroventral periventricular nucleus (Gu et al., 1996). Recently, the involvement of cAMP and protein kinase A (PKA) has been noted in estradiol-induced dendritic spine outgrowth in cultured hippocampal neurons (Murphy et al., 1998b). Estrogen also upregulates protein kinase C, and PKC-induced phosphorylation influences $Ca^{2+}$ currents and exocytosis (Drouva et al., 1988; Drouva et al., 1990; Fomina and Levitan, 1997). Estradiol has likewise been shown to elevate PKC catalytic activity in the preoptic area of female rats (Ansonoff and Etgen, 1998). Thus, $Ca^{2+}$ currents might be jointly regulated by estrogenic influences on transcription and phosphorylation.

Furthermore, experiments investigating the rapid effect of estrogen have demonstrated that estrogen can potentiate kainate-induced currents via activation of the cAMP cascade *in vivo* (Gu and Moss, 1996) and *in vitro* (Gu and Moss, 1998). These experiments have subsequently been completed in isolated hippocampal neurons of the estrogen receptor knock-out (ERKO) mouse (Gu et al., 1999). The data suggest that the potentiation of kainate-induced currents by 17β-estradiol was likely a G-protein coupled, cAMP-dependent phosphorylation event. In other experiments, estrogen dramatically enhanced expression of the neurotensin/neuromedin gene in preoptic neurons, even

though its promoter lacks an estrogen response element (Alexander and Leeman, 1994). This is the first experiment to show that estrogen has a direct effect on a neurotransmitter. More recently, it has been shown that estrogen regulates Neurotensin gene transcription by interactions with the cAMP/PKA cascade in SK-N-SH cells, a neuronal cell line (Watters and Dorsa, 1998). Neurotensin, a 13-amino acid peptide containing CREB and AP-1 recognition sites, is involved in stimulation of prolactin release and may participate in the preovulatory leutenizing hormone surge (Alexander and Leeman, 1994). It thus appears that estrogen has non-transcriptional roles in neuroendocrine function by modulating the expression of a neurotransmitter gene. These non-transcriptional effects, detected within seconds or minutes of estrogen exposure, are much too rapid for a genomic mechanism of action and indicate that estrogen may act on membrane receptors or other cellular components to alter neuronal events.

### *The Estrogen receptor is expressed in two forms, ERα and ERβ*

Recently, a novel estrogen receptor, called ERβ, was cloned (Kuiper et al., 1996) which is highly homologous to the original ER receptor, now termed ERα. The DNA-binding domain is 95% identical and the C-terminal ligand-binding domain is 55% identical (fig. 9). Like ERα (67kDa), ERβ (~54kDa) has a high affinity for estradiol and is capable of activating the transcription of an estrogen response element reporter gene construct (Kuiper et al., 1996). Studies of the regional distribution of ERα and ERβ indicate areas of overlap as well as some considerable differences. In some brain regions,

ERβ appears to compose a significant portion of total ER, and as such may play an important role in estrogen action. Differences exist in the ligand binding affinity and the ligand specificity of the ER subtypes, thus creating the potential for regional variations in activity. Estradiol has been shown to induce differential neuronal phenotypes by activating estrogen receptors α or β as well (Patrone et al., 2000). In this study, following transient transfection in neuroblastoma cells, ERα activation resulted in an increased length and number of neurites, whereas ERβ activation modulated only neurite elongation. Similarly, the hippocampus responds robustly to estrogen, and ERβ and ERα mRNA transcripts have been detected in the rat hippocampus (Shughrue et al., 1997) and the hippocampus of an ovariectomized female

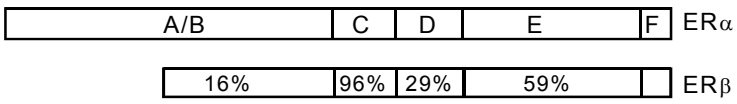



FIG 9: COMPARISON BETWEEN THE ERα AND ERβ SEQUENCES
Percentage amino acid identity in the domains A/B (N-terminus), C (DNA binding), D (hinge region), E ( ligand binding domain), and F (ligand dependent transactivation) are depicted.

monkey at a relatively high ERβ/ERα ratio (Register et al., 1998). Additionally, it has been demonstrated that some hippocampal pyramidal cells may express ERβ (Li et al., 1997b). These results suggest that ERβ may also play a role in mediating estrogen

effects in the mammalian hippocampus. The existence of at least two ERs and their possible co-localization in some brain regions and differential effects on gene expression expands the potential signaling pathways that could be involved in mediating genomic effects of estradiol. Finally, the functional impact of estrogen receptor localization in the hippocampus may prove relevant to the emerging role for estrogen as a protective factor in neurodegenerative injury. This potential role is further highlighted by the recent findings that the expression of ERα and ERβ changes following ischemic brain injury and that these changes correlate with the hormonal modulation of neuroprotection (Dubal et al., 1999).

### *Estrogen receptor α and β messenger RNAs are variably spliced*

Analysis of mRNA prepared from a variety of estrogen-responsive cell lines, breast tumor specimens, and normal breast tissue have established that estrogen receptor α mRNA is typically expressed as a mixture of transcripts (Miksicek et al., 1993; Gotteland et al., 1995; Poolaab et al., 2000). Analysis of these transcripts have revealed that the heterogeneity is largely a result of an imprecise pattern of mRNA splicing that gives rise to a family of correctly processed and exon-skipped transcripts. Although it has been proposed that the alternative spliced forms of ERα might be clinically significant in the progression of breast cancer and the resistance to therapy (Pfeffer et al., 1996), their existence in brain tissue is still debated. Similarly, ERβ mRNA has been reported to be variably spliced (Fig 10). The wild-type form has been designated ERβ1,

an 18 amino acid insert between exon 5 and 6 in the ligand binding domain (LBD) has been termed ERβ2, and a deletion of the second zinc finger domain of exon 3 has been

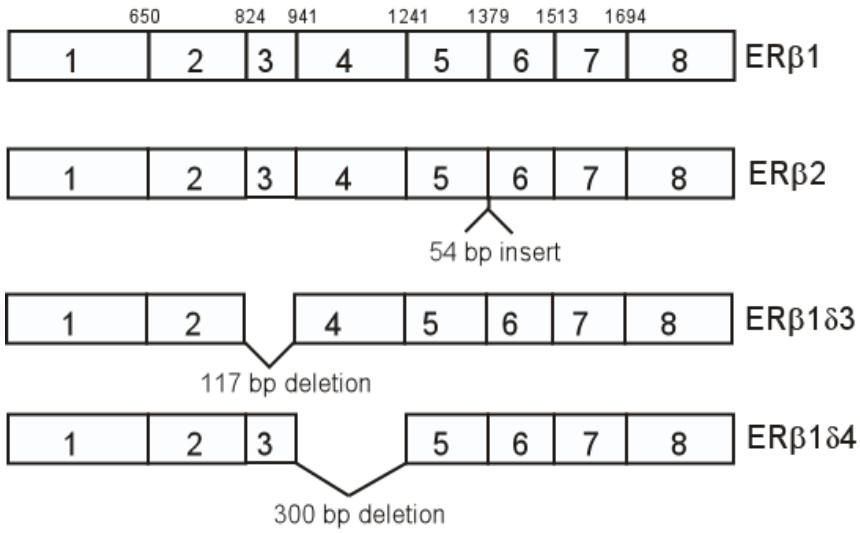



FIG 10:SCHEMATIC DIAGRAM OF ERβ mRNA SPLICE VARIANTS. Messenger RNA for ERβ is variably spliced resulting in proteins with altered function. The major variants are shown here along with the wildtype ERβ1 form. Each of these variants can also exist in combination with the others, producing a large combination of isoforms.

termed ERβ1δ3. A combination of these two variants, then, produces ERβ2δ3. Furthermore, recent work in our lab has resulted in the identification of a novel, naturally occurring ERβ mRNA splice variant that is missing exon 4 and has been designated ERβ1δ4 (Price et al., 2000). It also appears that all of these variants can be translated into functional proteins, each of which has altered pharmacological properties. Therefore, splice variation of steroid receptor mRNA can lead to alteration of receptor function

depending on which portion of the receptor is altered by the insertion or deletion. Given that many ERβ mRNA splice variants are expressed in various normal rat tissues at levels equal to those of the wild type form, these variants should be considered in addition to ERβ1 and ERα when describing the effects estrogen.

*Co-activators and co-repressors influence estrogen receptor transcriptional activation*

The nuclear hormone receptors are transcriptional regulators that activate gene transcription upon binding of their respective ligands. While there is considerable evidence that nuclear receptors can contact some of the basal factors of the preinitiation complex directly, without the need for intermediary coregulatory proteins, evidence for additional factors is supported by observations that different classes of receptors can interfere with one another's transcriptional activity by squelching limiting factors that are not components of the basal transcription machinery (Meyer et al., 1989).

Recent data show that nuclear receptors enhance or inhibit transcription by recruiting an array of proteins to the transcription complex. These have been termed coregulatory proteins collectively, and can be subdivided into coactivators, corepressors, and integrators. Coactivator proteins interact directly with the activation domain of a nuclear receptor in an agonist-dependent manner (but not in the presence of an antagonist), leading to enhancement of the receptor activator function. A number of biochemical and yeast two-hybrid studies have identified proteins capable of interacting with NRs and influencing their transcriptional activity. Transcriptional activation by both AF-1 and AF-2 of the estrogen receptor is cell type specific and relies on the promotor

context of the hormone response element (Tora et al., 1989). This suggests the existence of different mediating or coactivating proteins, several of which have been identified to date.

Two distinct steps in target gene activation appear to be regulated by coactivators. First, they play a role in remodeling chromatin, a process that involves the destabilization of histone-DNA contacts to allow the binding of other transcription factors (Kadonaga, 1998). Second, coactivators are required to recruit the transcription machinery, ultimately RNA polymerase II, to transcribe target genes. This involves the recruitment of a protein complex that interacts with one or more subunits of the polymerase and is essential for transcriptional initiation.

Over 30 potential coactivators have been identified by their ability to bind various receptor domains and to alter the transcriptional activity of nuclear receptors (McKenna et al., 1999a; Glass and Rosenfeld, 2000). These coactivator proteins function as signaling intermediates between the receptors and the general transcriptional machinery to affect gene transcription (see Horwitz et al., 1996; Katzenellenbogen et al., 1996). Distinct classes of ligand-dependent transcriptional cofactors have been described, including CBP/p300 and the p160 family (Xu et al., 1999). At present, the p160 family contains three members including SRC-1/N-CoA1 (Onate et al., 1995; Kamei et al., 1996), SRC-2/GRIP-1/TIF2 (Hong et al., 1996; Voegel et al., 1996) and SRC-3/AIB1/p/CIP (Torchia et al., 1997, Li, 1997, Suen, 1998). The p160 proteins contain a short conserved nuclear receptor interaction motif (NR box), which has the core sequence LXXLL (L = leucine and X = any amino acid) (Heery et al., 1997; Torchia et al., 1997).

All of the p160 coactivators have a core NR interaction domain that contains three of these NR boxes arranged in tandem. A unique fourth NR box has been characterized at the extreme carboxyl terminus of an alternatively spliced variant of SRC-1, SRC-1a (Takeshita et al., 1996). Moreover, the p160 family is defined by an overall sequence similarity of 40% between the three proteins, distinguishing its members from other coregulator classes. The highest degree of homology is in the N-terminal domains, where the basic helix-loop-helix (bHLH) domain exhibits a high degree of similarity. This bHLH domain is responsible for mediating protein-protein interactions with other transcriptional machinery.

How these proteins function as coactivators is currently speculative, but attention centers on their interactions with the basal transcription machinery and on their associated histone acetyltransferase (HAT) activity. Both CBP and SRC-1 bind to TATA-binding protein (TBP) (Abraham et al., 1993; Takeshita et al., 1996), and the TBP-binding site of CBP serves as an independent activation domain when brought to DNA (Swope et al., 1996). These mediators interact with the LBD and some are capable of increasing the AF-2 response in a ligand-dependent manner. On certain promoters, AF-1 and AF-2 must synergize to reach efficient activation. Additionally, while coactivators interact with components of the basal transcription machinery they don't enhance the basal transcriptional activity by their own. Coactivators may mediate interactions with other transcription factors and play a role in the assembly and stabilization of the transcriptional preinitiation complex.

TABLE 1

| Cofactor | Alternative Designations | Related Factors | Notes | References |
|---|---|---|---|---|
| ERAP-160 | GRIP-170, p160 | ERAP-140 | ERAPs bind ER in ligand-dependent manner; mammalian cellular fraction enriched in GRIP-170; coactivates GR | (Halachmi et al., 1994) |
| SRC-1 | hSRC-1<br>NcoA-1/<br>mSRC-1<br>P160 | TIF2/<br>hSRC-2/<br>mSRC-3<br>hSRC-3 | Interacts with and coactivates nuclear receptors; interacts with CBP/p300; contacts basal transcription factors; possesses acetyltransferase activity, intareacts with PCAF; contains autonomous activation domains; targeted deletion causes hormone insensitivity | (Onate et al., 1995; Kamei et al., 1996) |
| TIF2/<br>hSRC-2 | GRIP-1/<br>mSRC-2<br>NcoA-2, p160 | hSRC-1<br>mSRC-3/<br>hSRC-3 | Interacts with and coactivates nuclear receptors; interacts with CBP | (Hong et al., 1996; Voegel et al., 1996; Torchia et al., 1997) |
| p/CIP/<br>mSRC-3 | ACTR/RAC3<br>AIB-1/TRAM-1<br>hSRC-3/p160 | SRC-1<br>TIF2/<br>hSRC-2<br>GRIP-1 | P/CIP coactivates CBP-mediated signaling pathways<br>AIB-1 interacts with, coactivates ER; overexpressed in breast tumors<br>TRAM-1 interacts with and coactivates TR<br>SRC-3 preferentially coactivates ERα over ERβ | (Torchia et al., 1997; Xu et al., 2000) |
| ARA-70 | | | ARA-70 interacts with and coactivates AR in prostate cells | (Yeh and Chang, 1996) |
| CBP | | p300 | Interacts with and coactivates multiple activators, including nuclear receptors; acetyltransferase; interacts with PCAF, SRC-1, TIF2/hSRC-2 and p/CIP mSRC-3 | (Chakravarti et al., 1996; Kamei et al., 1996) |
| p300 | | CBP | Broad functional similarity to CBP; interacts with and coactivates nuclear receptors; possesses acetyltransferase activity; interacts with PCAF, SRC-1 and p/CIP/mSRC-3 | (Kwok et al., 1994) |
| PCAF | hGCN5 | | Possesses intrinsic acetyltransferase activity; interacts with PR and SRC-1; interacts with TR and ACTR | (Blanco et al., 1998; Kotani et al., 1998; Krumm et al., 1998) |

Originally, it was considered that the inhibitory activity of steroid receptor antagonists was due simply to the competition between agonists and antagonists for binding to the receptor. However, there is increasing evidence that suggests that antagonists activity results from an active process which involves the recruitment of transcriptional corepressors (Horwitz et al., 1996; Xu et al., 1996; Webb et al., 1999). Two corepressors have recently been identified: a 270-kDa mouse protein termed N-CoR (nuclear receptor corepressor; (Horlein et al., 1995) and a 168-kDa human protein termed SMRT (silencing mediator for retinoid and thyroid hormone receptors; (Chen et al., 2001), both of which have similar properties. SMRT, N-CoR, and their homologs represent a new class of transcriptional mediators for nuclear receptors that actively silence basal transcription. As with coactivators, the receptor/corepressor dyad may be a subunit of an even larger inhibitory complex, since there is evidence that the repressive effects of unliganded steroid receptors result not only from binding a corepressor, but also from the ability to interact with and sequester or inactivate TFIIB directly (Baniahmad et al., 1993).

Therefore, in the absence of hormone, unliganded receptors interact with a family of transcriptional corepressors, including SMRT and N-CoR, which target histone deacetylases to establish a condensed and repressed chromatin structure. Upon hormone binding, the corepressor complex is replaced by a coactivator complex containing SRC1, CBP and/or GRIP or SRC-2, and this is followed by transcriptional initiation.

*ERs are transiently expressed in the hippocampus during development*

The classical intracellular estrogen receptor has been identified in the rat hippocampus by several different methods, including *in situ* hybridization and immunocytochemistry (Pfaff and Keiner, 1973; Loy et al., 1988; Pelletier et al., 1988; Maggi et al., 1989). Work in our laboratory has shown that ER and ER mRNA are found at high levels in the hippocampus during the first postnatal week of development, with a subsequent decline to the adult level (O'Keefe and Handa, 1990; O'Keefe et al., 1995; Solum and Handa, 2001). Within the adult hippocampus, estrogen receptors are primarily found in interneurons (Weiland et al., 1997), although recent studies have demonstrated the presence of ERs in pyramidal cells as well (Shughrue and Merchenthaler, 2000). In contrast, in the neonate, ERα is predominantly distributed throughout the pyramidal cell layer (Solum and Handa, 2001), suggesting its presence in pyramidal cells as well as interneurons. Within the pyramidal cell layer, the highest density is found in the CA3 region, intermediate levels are found in CA1, and lower levels are found in the dentate gyrus (O'Keefe et al., 1995; Solum and Handa, 2001). Furthermore, other investigators have demonstrated the presence of ERs in hippocampal cells *in vitro* (Murphy et al., 1998b). Preliminary data from our laboratory have shown transient increases in both ERα and ERβ mRNA in the developing brain as well (Price Jr. and Handa, 1997). Unfortunately, most studies of the effects of estrogen in the hippocampus have been conducted in the adult, not the neonate.

*Functional Significance of ER in adulthood*

Estrogen has been shown to influence a variety of processes associated with hippocampal function. In addition to altering learning behavior (O'Neil et al., 1996) estrogen facilitates kindled seizures in the hippocampus (Buterbaugh and Hudson, 1991) and has the ability to decrease seizure threshold in the hippocampus (Terasawa and Timiras, 1968). Studies have shown that while transynaptic responses of pyramidal cells in CA1 are not affected by changes in circulating estrogen following low-frequency stimulation, synaptic plasticity, which is at the basis of Hebbian associative memory, is facilitated by estrogen treatment (Cordoba Montoya and Carrer, 1997). Research by Warren (Warren et al., 1995) has found that induction of long-term potentiation (LTP), which depends on synaptic plasticity, is maximal in female rats during the afternoon of proestrus. It has been known for some time that synaptic plasticity and LTP in CA1 depend on the availability of NMDA type glutamate receptors (Collingridge and Singer, 1990), and coincidentally in the adult, estrogen increases the number of NMDA type binding sties in CA1 (Weiland, 1992a). Woolley and colleagues (Woolley et al., 1997) recently confirmed this finding and proposed that this increased affinity may be explained by the greater concentration of the NMDAR1 receptor protein that is regulated by estradiol. These changes in NMDA binding result in greater sensitivity to NMDA receptor–mediated synaptic input. This effect was demonstrated in slices taken from estrogen treated rats in which responses to AMPA receptor-mediated synaptic input and GABA-mediated inhibition were blocked (Woolley et al., 1997). An inquiry into the functional consequences of these changes using intracellular recording showed that

estradiol administration increases synaptic excitability and prolonged the EPSP in 20% of CA1 pyramidal neurons (Wong and Moss, 1992) and markedly increased the amplitude of NMDA mediated EPSPs (Foy et al., 1999). In extracellular recording experiments, slices perfused with 100pM of 17β-estradiol exhibited a pronounced, persistant, and significant enhancement of LTP of both the fEPSP slope and amplitude compared to controls 30 minutes after high frequency stimulation (Foy et al., 1999). The dynamic regulation of plasticity by estrogen may also be operative in the ventromedial hypothalamic nucleus (Frankfurt et al., 1990), arcuate nucleus (Matsumoto and Arai, 1979), lateral septal nucleus (Miyakawa and Arai, 1987), and preoptic area (Langub et al., 1994) where the steroid has been shown to modulate the number or size of synaptic contacts.

Ovarian hormones also regulate the synaptic connectivity of hippocampal neurons. The hippocampal synapses regulated by estradiol are excitatory synapses formed on dendritic spines. Experiments have demonstrated that dissociated hippocampal neurons grown in culture can double their dendritic spine density in response to estradiol (Murphy and Segal, 1996), confirming earlier in vivo experiments (Gould et al., 1990; Woolley and McEwen, 1992). As glutamate is the primary neurotransmitter at CA1 pyramidal cell dendritic spine synapses, it follows that glutamate receptor numbers increase in parallel with spine synapses (Weiland, 1992b; Woolley et al., 1997). The combined morphological and electrophysiological analysis of estradiol's effect on CA1 pyramidal cells suggests that estradiol-induced spine synapses are a structural substrate of the estradiol induced increase in synaptic sensitivity to NMDA

receptor-mediated input. An alternative explanation is derived from data showing that estrogen is acting by altering levels of GABA neurotransmission (Murphy et al., 1998b). This work demonstrated *in vitro* that estrogen decreases the levels of glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme. The resulting effect is a decreased inhibition suggesting that in the adult synaptic excitability is facilitated by disinhibition of pyramidal cells instrumented through the action of estradiol on inhibitory interneurons, since estrogen is preferentially taken up by interneurons (Loy et al., 1988). It has been suggested that the mechanism for this estradiol induced increase in dendritic spine density *in vitro* is through the phosphorylation of cAMP response element binding protein (CREB); (Murphy and Segal, 1997). More recent *in vivo* experiments showed that ovariectomy decreases GAD levels and estrogen protects against this decrease (Hart et al., 1999). In a further attempt to determine which hippocampal neuronal populations are activated by estradiol, (Rudick and Woolley, 1999) analyzed the induction of c-Fos immunoreactivity following estrogen treatment. This study found that the density of c-Fos nuclei in pyramidal cells, but not interneurons, is increased within 2 hours of estradiol treatment, suggesting a direct effect on pyramidal cells. Estrogen also increases the expression of the transcription factor c-*fos* in the CA1 region (Jennes, 1990), further indicating that estrogen has activational effects on hippocampal physiology.

### *Effects of Estrogen on Memory and Cognition*

Accumulating evidence from a variety of experimental human (Phillips and Sherwin, 1992; Kampen and Sherwin, 1994; Kimura, 1995), and animal (Luine and Rodriguez,

1994; Diaz-Veliz et al., 1995; Kampen and Sherwin, 1996; O'Neil et al., 1996; Packard et al., 1996) data indicate that estradiol may affect aspects of brain chemistry and morphology known to be important for memory functions. Prospective, controlled studies of surgically and naturally menopausal women demonstrated that exogenous estrogen enhanced short- and long-term memory and the capacity for learning new associations, whereas visual memory was unaffected. The hormonal influences on memory processes appear to involve action on brain structures such as the hippocampus and basal forebrain.

Animal studies have suggested that estradiol influences performance on various learning and memory tests. In male rats, acquisition of radial maze behavior is impaired by neonatal gonadectomy or administration of the aromatase inhibitor androst-1,4, 6-triene-3,17-dione, indicating an organizational effect on spatial behavior exerted by the aromatization of testosterone to estradiol (Williams et al., 1990).

In humans, a role for estrogen in memory is suggested from studies examining the effects of hormone replacement therapy in postmenopausal women. For example, estrogen replacement therapy enhances immediate and delayed verbal recall in both naturally and surgically menopausal women (Sherwin, 1994).

### *Estrogen and Neuroprotection*

A significant number of studies have clearly established that estradiol is a potent neuroprotective and neurotrophic factor for neurons. It is no surprise then, that the decline of ovarian activity after surgical and natural menopause appears to affect a number of brain structures, including mood and certain types of memory.

Epidemiological evidence suggests that estrogen replacement therapy for postmenopausal women is associated with an improvement of some measures of cognitive performance, protection against cognitive deterioration, and decreased incidence of Alzheimer's disease (Paganini-Hill and Henderson, 1994; Tang et al., 1996; Sherwin, 1997; Costa et al., 1999). Additionally, beneficial effects of estrogen on the mortality and morbidity associated with cerebral stroke have been demonstrated (Lafferty and Fiske, 1994; Grodstein et al., 1996; Hurn and Macrae, 2000). .

The protective effects of estrogen on different cell damaging factors have been described *in vitro* and *in vivo* (see (Wise et al., 2000; Garcia-Segura et al., 2001). The first studies of the neuroprotective effects of estradiol were detailed in relation to the effects of serum deprivation on neuronal survival in cell culture (Arimatsu and Hatanaka, 1986). Since then, protective effects of estradiol have been reported in 12 different types of neuronal cells against 14 different toxicities including serum deprivation (Bishop and Simpkins, 1994; Green et al., 1997; Gollapudi and Oblinger, 1999), oxidative stress (Behl et al., 1995; Goodman et al., 1996; Sawada et al., 1998), amyloid β pepdtide (Aβ) induced toxicity (Goodman et al., 1996; Green et al., 1996; Mattson et al., 1997) and excitotoxicity (Singer et al., 1996; Regan and Guo, 1997; Weaver et al., 1997). These studies demonstrated that physiological and pharmacological doses of 17β-estradiol profoundly attenuate the extent of injury and decrease neuronal cell death.

However, fewer studies have examined the neuroprotective effects of estrogen with animal models (see McEwen and Alves, 1999; Garcia-Segura et al., 2001). Of these it has been demonstrated that in the female rat, physiological levels of estradiol protect

against ischemic damage induced by middle cerebral artery occlusion (Simpkins et al., 1997; Alkayed et al., 1998; Dubal et al., 1998). Moreover, in estrogen receptor α knock-out mice, estradiol did not protect the brain against ischemic injury, indicating that ERα is a critical link in estradiol-mediated neuroprotection (Dubal et al., 2001). The discovery that ERα mediated protection of the brain carries far-reaching implications for the selective targeting of ERs in the treatment and prevention of neural degeneration.

Although estrogen is a well-described neuroprotective agent, the mechanism by which estrogens exert their neuroprotective actions is less clear. Recent studies attempting to determine estrogen's mechanism of action in neuroprotection revealed that in response to injury, estrogen modulates critical factors such as bcl-2 (Singer et al., 1998; Dubal et al., 1999; Pike, 1999) and immediate early genes.

It appears that estradiol can act via mechanisms that require classical intracellular receptors (estrogen receptor alpha or beta) that affect transcription, via mechanisms that include cross-talk between estrogen receptors and second messenger pathways, and/or via mechanisms that may involve membrane receptors or channels. Additionally, estrogens exert protective effects on neuronal cells in culture that may be mediated, at least in part, by their ability to alter free radical production and/or free radical action on cells or by suppressing oxidative stress in neurons (Behl et al., 1995; Mattson et al., 1997). Finally, the neuroprotective actions of estrogen could be mediated, at least in part, through interactions with the previously described neurotrophins. By influencing the expression

of these trophic factors and/or their receptors, estrogen could profoundly influence the survival of neurons in both the developing and adult brain.

### *Estrogen Receptors are Co-expressed with the Neurotrophins*

The neurotrophins are a family of growth factors including BDNF, NGF, NT-3, NT-4/5 and the fish neurotrophin, NT-6. Like estrogen, the neurotrophins have important influences on the development, survival, plasticity, and aging of neurons in mammalian brain regions that subserve reproductive, cognitive, and various other functions. The biological activities of the neurotrophins are mediated by two structurally distinct classes of cell membrane receptors that are preferentially expressed in neural tissues (Chao, 1992; Raffioni et al., 1993; Chao and Hempstead, 1995). One class consists of members of the tropomyosin-related kinase (*trk*) family of receptor tyrosine kinases *(trkA, trkB, and trkC*), each of which mediate neurotrophin signaling through increased tyrosine autophosphorylation of its cognate receptor. NGF binds *trkA*; BDNF binds to *trkB,* as does NT-4/5 but with lower affinity; and NT-3 binds primarily to *trkC*. The second receptor, $p75^{NTR}$, is a pan-neurotrophin receptor that binds all neurotrophins with low affinity. The *trks* can mediate responses to the neurotrophins with or without the participation of $p75^{NTR}$. Neurotrophin binding to Trk receptors induces receptor dimerization, autophosphorylation and generation of a signaling cascade resulting in neuronal differentiation and survival. Interestingly, neurons in forebrain regions of both sexes in the rat coexpress estrogen and neurotrophin receptors and are the sites of estrogen and neurotrophin synthesis (Toran-Allerand et al., 1992; Miranda et al., 1993b),

suggesting a potential interaction between these two receptor systems. In addition, estrogen receptor and neurotrophin receptor co-expression leads to convergence or cross-coupling of their signaling pathways (Toran-Allerand et al., 1999). However, an important and unanswered question

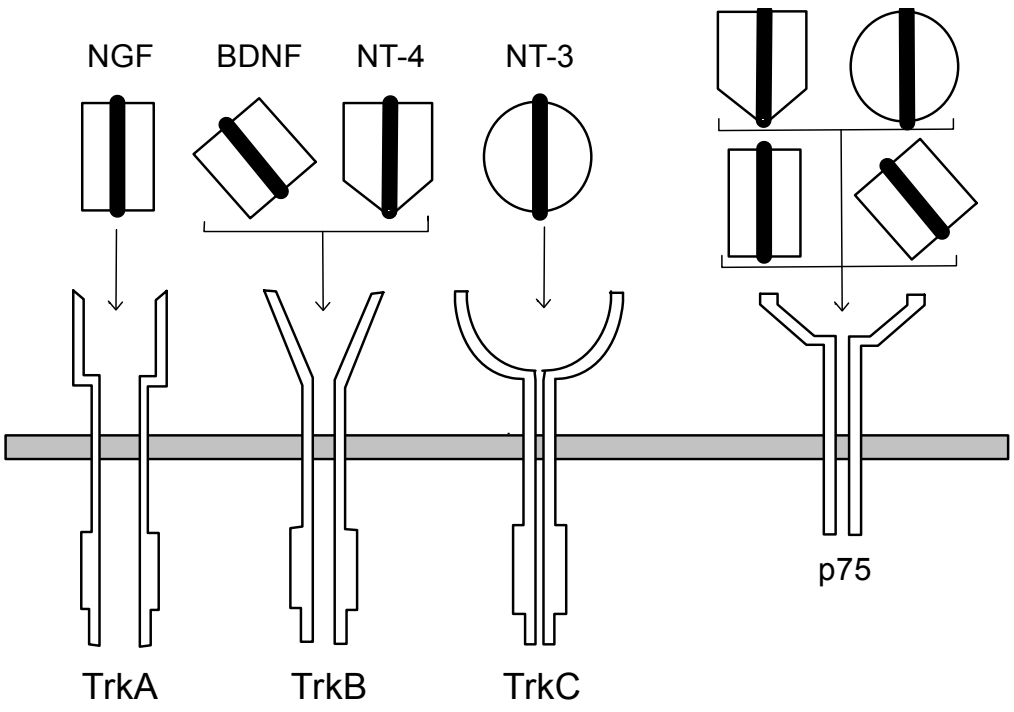



Figure 11: INTERACTIONS BETWEEN NEUROTROPHINS AND THEIR RECEPTORS. All Neurotrophins bind to the low affinity p75 receptor, whereas binding to the high-affinity Trks is specific to the different neurotrophins. NGF binds to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC.

is whether the developmental actions of estrogen on neurite growth and differentiation are mediated directly or result from intermediate steps via modulator interactions with endogenous growth factors and their signaling pathways.

The neurotrophic effects of gonadal hormones appear to play some role in the developmental determination of sex differences as well. For example, a role for nerve growth factor (NGF) in the sexual differentiation of the brain is suggested by evidence that the administration of NGF antibodies to neonatal rats prevented testosterone-mediated defeminization of sexually dimorphic reproductive behavior and excitability of VMN-midbrain projections (Yanase et al., 1988). Similarly, anti-NGF attenuated the estrogen-mediated defeminization of lordosis (Hasegawa et al., 1991).

### *Function of the Neurotrophins*

According to the classic mechanisms of neurotrophin action, neurotrophins are released constitutively and influence the development and survival of certain populations of neurons, depending on the specific Trk receptors they express. Such mechanisms operate in almost all regions of the nervous system and have been studied extensively during the past few decades (Barde, 1989; Davies, 1994). It also appears that neurotrophins can influence synaptic transmission at both developing and adult synapses. In addition to their specific patterns of expression in the adult CNS, the neurotrophins and their receptors are developmentally regulated (reviewed by (Davies, 1994). BDNF, NT-3, and NT-4/5 mRNA levels increase in abundance with postnatal age (Friedman et al., 1991; Timmusk et al., 1993), and in rats, mRNA levels for TrkB and TrkC transiently

peak between postnatal day (P1) and P14 in several different brain regions, correlating with maximal neuronal growth, differentiation, and synaptogenesis (Ringstedt et al., 1993; Altar et al., 1994; Knusel et al., 1994). Furthermore, McKay and colleagues (Vicario-Abejon et al., 1998) have shown that neurotrophins can speed up the development of both excitatory and inhibitory synaptic transmission in cultured hippocampal neurons. Moreover, neurotrophins have been shown to promote neuronal survival during development and after various brain insults (Lindvall et al., 1994). In fact it has been suggested that the trophic support provided by the neurotrophins is *required* for nerve growth during development (Tucker et al., 2001).

Additionally, a further function can be assigned to neurotrophins. Recent evidence has accumulated that neurotrophins might serve as feedback regulators for the efficacy of synaptic transmission in the mammalian CNS. It was shown that neurotrophins are synthesized in an activity dependent manner by neurons and are released upon depolarization (Thoenen, 1995; McAllister et al., 1999), making them ideally suited to influence the neuronal activity in an autocrine fashion. In the hippocampus, changes in BDNF mRNA levels are particularly dramatic, increasing by over six fold in dentate granule cells within 30 minutes after seizure induction (Ernfors et al., 1991). Additionally, in the developing brain, kainic acid or pilocarpine induced seizures resulted in dramatic increases of BDNF mRNA in the hippocampus and cortex as early as postnatal day 7 (Kornblum et al., 1997). Similarly, manipulations meant to mimic neuronal activity are particularly effective in regulating neurotrophin mRNA levels in dissociated neuron cultures. Depolarization of cultured hippocampal neurons

and cerebellar granule cells with glutamate receptor agonists or high potassium, for example, dramatically increases the levels of mRNAs encoding BDNF and NGF (Bessho et al., 1993; Lindholm et al., 1994; Berninger et al., 1995). Conversely, BDNF and NGF levels are down regulated in the presence of GABA through activation of $GABA_A$ receptors (Zafra et al., 1991; Berninger et al., 1995). This specific spatial and temporal distribution of the neurotrophin and Trk receptors indicates that they are likely to be in the right place at the right time to be involved in both developmental and adult plasticity. Additionally, these studies suggest that brain derived neurotrophic factor acting through its receptor trkB may be involved in protection mechanisms after damage during seizures as well as in sprouting responses.

### *Brain-Derived Neurotrophic Factor*

Brain-derived neurotrophic factor (BDNF) is a 27-kDa polypeptide that is recognized to play an important role in the survival, differentiation, and outgrowth of select peripheral and central neurons during development and in adulthood (Conover and Yancopoulos, 1997; Lu and Figurov, 1997). The rat BDNF gene gives rise to four major transcript forms, and each contains a unique 5' exon (I-IV) and a common 3' exon (V) that codes for the mature BDNF protein (Timmusk et al., 1993). BDNF binds with high affinity to a specific receptor tyrosine kinase, TrkB (Klein et al., 1991), both of which are widely expressed in the central nervous system. In particular, the hippocampus expresses high levels of mRNA for BDNF (Hofer et al., 1990) and the mRNA for TrkB is found on

both pyramidal and non-pyramidal hippocampal neurons (Ip et al., 1993; Barbacid, 1994).

The traditional role of neurotrophins as retrogradely transported, target-derived survival factors is well established in the developing nervous system (Levi-Montalcini, 1987). For instance BDNF is produced by targets of BDNF-dependent neurons in

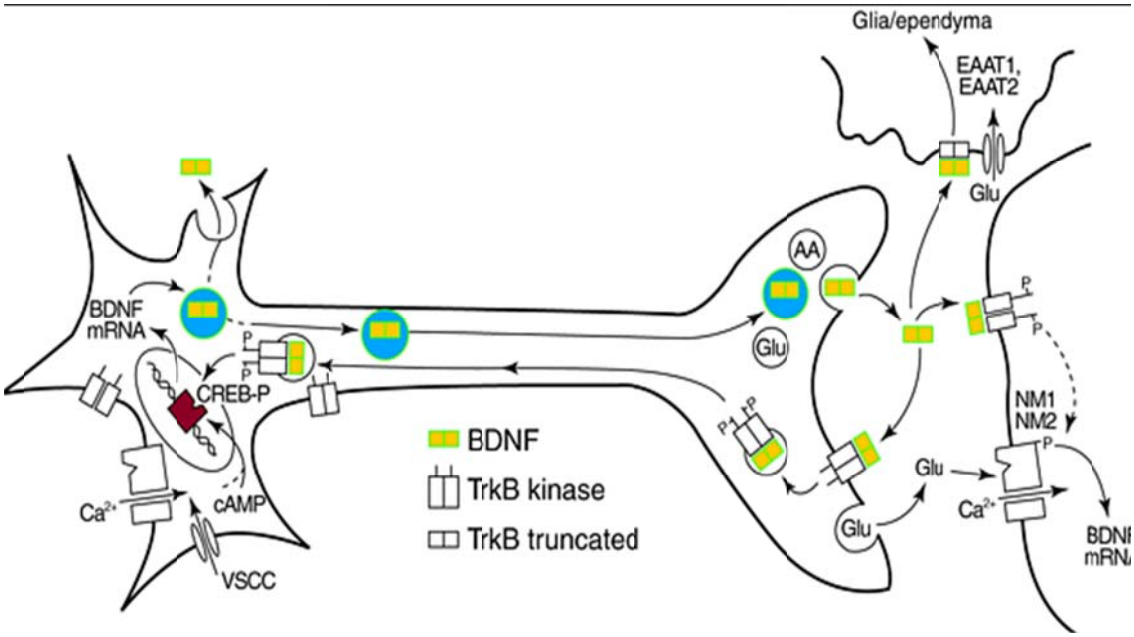



FIG 12: BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) FULFILLS THE CLASSICAL CRITERIA FOR BEING CONSIDERED AS A RAPIDLY ACTING SYNAPTIC MODULATOR. These criteria are illustrated for a typical axodendritic connection between glutamatergic and GABAergic interneurons. CREB-P, phosphorylated cyclic AMP response-element-binding protein; EAAT, excitatory amino acid transporter, Glu, glutamate, NM1, NM2, NMDA receptor subunits 1 and 2; P, phosphate; TrkB, BDNF receptor; VSCC, voltage-sensitive $Ca^{2+}$ channel. Modified from Altar, (1998).

factors are functionally similar to retrogradely acting growth factors such as nerve limiting quantities and promotes neuron survival through its retrograde transport and signaling to the cell body. Recent studies have shown that the endogenous neurotrophins, BDNF and NT-3, are transported anterogradely by central and peripheral neurons. The supply of BDNF by afferents is consistent with their presynaptic growth factor (see Levi-Montalcini, 1987). BDNF has also been shown to play an tissues, where they function as trophic factors and act similar to neurotransmitters (Altar et al., 1997). This suggests that these anterogradely transported and released trophic synthesis, vesicular storage release and postsynaptic actions (Fig. 8). Anterograde axonal transport provides an afferent supply of BDNF and NT-3 to neurons and target important role in activity-dependent synaptic plasticity in the hippocampus (Kang and Schuman, 1995; Korte et al., 1995; Korte et al., 1996; Patterson et al., 1996), to produce a lasting potentiation of synaptic efficacy in the dentate gyrus (Messaoudi et al., 1998), and to enhance glutamatergic synaptic transmission in hippocampal cultures through a presynaptic mechanism (Li et al., 1998). In addition, TrkB ligands play a role in the visual cortex to regulate development of ocular dominance columns (Cabelli et al., 1997), in sculpting the dendritic morphology of pyramidal neurons, and in enhancing synaptic transmission (Akaneya et al., 1997). Similar effects have been demonstrated in hippocampal cell cultures, where BDNF has been shown to enhance growth and synaptic activity (Bartrup et al., 1997). BDNF also induces the formation of excitatory and inhibitory synapses (Vicario-Abejon et al., 1998; Bolton et al., 2000) and enhances the release of neurotransmitters (Li et al., 1998). In slices from the developing hippocampus several

studies have found that the application of BDNF influenced paired-pulse facilitation (Kang and Schuman, 1995; Gottschalk et al., 1998).

The production of BDNF has also been shown to be regulated by synaptic activity. Induction of hippocampal long-term potentiation (LTP), for example, rapidly and selectively increases BDNF mRNA levels, with little or no effect on the other neurotrophins (Castren et al., 1993; Dragunow et al., 1993). More recently, it has been demonstrated that BDNF is upregulated during declarative memory formation in primates (Tokuyama et al., 2000) suggesting that BDNF contributes to reorganization of neuronal circuits for long-term memory formation. Furthermore, experiments have shown that BDNF is rapidly and selectively induced in the hippocampus during contextual learning (Hall et al., 2000) and spatial memory formation and maintenance (Mizuno et al., 2000). The increase in hippocampal BDNF expression during contextual learning further supports the suggestion that BDNF is involved in synaptic plasticity of the brain. Together, these studies indicate that BDNF can have an effect on established synaptic contacts that gives rise to the possibility that it can influence synaptic plasticity (Thoenen, 1995; Figurov et al., 1996).

### *Effect of Gonadal Hormones on BDNF*

As recently been reported in adult rats, relative levels of BDNF mRNA within specific regions of the hippocampus can fluctuate significantly over the course of the estrous cycle with lowest levels detected in CA1 and CA3 on the afternoon of proestrus (Gibbs, 1998). In addition, relative levels of BDNF mRNA in regions CA1, CA3 and the

dentate gyrus were significantly increased in ovariectomized rats treated with estrogen and progesterone relative to ovariectomized controls. Evidence for increased hippocampal levels of BDNF mRNA following long-term continuous estrogen treatment has also been reported (Singh et al., 1995). In addition, recent evidence demonstrates that soy phytoestrogens can regulate levels of BDNF (Pan et al., 1999). In this study, it was shown that following ovariectomy, levels of BDNF mRNA decreased in the frontal cortex and a diet with added soybean estrogens returned the levels to that of intact controls. Murphy et al. (1998a) recently demonstrated that short-term treatment with estradiol produces a significant decrease in BDNF protein in primary rat hippocampal cultures, and that the decrease in BDNF plays an important role in estrogen-mediated increases in spine density on CA1 pyramidal cells. One possibility for the observed down-regulation of BDNF by estrogen may actually be a result of increased transport of the neurotrophin as recently suggested (Gibbs, 1999). Modulation of BDNF levels by estradiol may operate at the transcriptional level because of the sequence homology existing between the nuclear estrogen response element (ERE) and regions within exon V of the BDNF gene (Sohrabji et al., 1995). Estrogen-bound receptor may bind to these regions to directly regulate BDNF expression levels. Alternatively, estrogens could affect BDNF expression indirectly through the activation of neurons that regulate BDNF or estrogen-responsive afferents to the target region.

Steroid hormones have been shown to affect the levels of BDNF in avians as well. For instance, testosterone treatment increases the levels of BDNF protein in the high vocal center (HVc) of the adult female canary (Rasika et al., 1999). These effects could

be mediated by estradiol, a metabolite of testosterone. In a similar system, it has been shown that the expression of BDNF mRNA is increased significantly with the HVc of male but not female zebra finches during development (Dittrich et al., 1999). Furthermore, premature stimulation on the normal increase of the BDNF mRNA expression in juvenile male zebra finches occurred after treatment with 17β-estradiol.

Given the increasing evidence for effects of BDNF on neuronal connectivity and activity-dependent synaptic plasticity in the adult brain, it is reasonable to hypothesize that hormonal effects on BDNF expression can contribute to effects on brain structure and function. In the hippocampus specifically, the up-regulation of BDNF expression could be a mechanism by which estrogen triggers the differentiation of cells within and connected to this brain region. It is possible then, that these effects will contribute to the effects of estrogen on cognitive processes and neuronal morphology.

### *Summary and Hypothesis*

The hippocampus is a tri-synaptic circuit in the brain that is involved in many functions, including spatial mapping, and learning and memory. Its development begins prenatally and follows a discrete timeline that continues well after birth. The development of the hippocampus may be influenced by estrogen, as suggested by morphological differences between males and females. It is currently unknown how estrogen mediates these effects on the developing hippocampus, but one possible way is by altering synaptic physiology. The developmental actions of estrogens may be mediated directly or indirectly through autocrine responses or local paracrine

mechanisms involving interactions with growth factors or steroid receptor coregulatory proteins. Through these interactions, estrogen may act to enhance the excitatory environment of the developing hippocampus. This enhanced level of excitation during development is thought to provide trophic support and alter intracellular calcium levels. The inability of steroids to exert an organizing effect on the brain after the critical period of sexual differentiation may be functionally related to hormonally mediated changes in neurotrophin expression during development.

Furthermore, the mechanisms of estrogen signaling have become increasing complex with the recent cloning of ERβ and the realization that ERs may interact with coregulatory proteins to affect transcription. Our preliminary data showing alterations in the expression of BDNF mRNA and protein in the hippocampus following early hormonal manipulations lead me to hypothesize that *during rat brain development, estrogen acts on the hippocampus by modulating brain derived neurotrophic factor, its receptor trkB, and/or the p160 coactivator proteins.* Based on the nuclear expression of ERα in neurons of the developing hippocampus, I believe that estrogen is having direct transcriptional effects to alter morphological and biochemical properties of the rat hippocampus.

# CHAPTER 3

# LOCALIZATION OF ESTROGEN RECEPTOR ALPHA (ERα) IN PYRAMIDAL NEURONS OF THE DEVELOPING RAT HIPPOCAMPUS


## *Abstract*

During development, estrogen has a variety of effects on morphological, biochemical and electrophysiological properties of hippocampal neurons. Correspondingly, estrogen receptor (ER) binding and mRNA increase transiently in the developing hippocampus. In this study, we used immunocytochemistry to determine the localization of the ERα subtype in the developing rat hippocampus. Nuclear staining was present in pyramidal cells and some interneurons of the CA1 and CA3 regions of the developing rat hippocampus. Little or no immunoreactivity was observed in postnatal day (P)0 animals (day of birth = P0), however, beginning on P4, ERα immunoreactivity (ERα-ir) was visible and reached maximal levels by P10. These levels subsequently declined to low levels so that by P15, levels approximated those of adult females. Western blot analysis confirmed that this antibody recognized a 67kd protein, characteristic of the full length ERα protein, in the hippocampus and pituitary. Furthermore, most of the ERα immunopositive cells in the hippocampus were located in the pyramidal cell layer, and did not co-localize appreciably with γ-amino butyric acid

(GABA) at any age examined. We conclude, based on the immunocytochemical localization of ERα, that the effects of estrogen on biochemistry and morphology of the developing hippocampus may be direct through the ERα subtype in hippocampal pyramidal cells.

## *Introduction*

In addition to their primary role in the maintenance and regulation of reproductive capacity, estrogens influence more general neurobiological functions, such as perceptual-spatial skills and learning and memory (McEwen, 1983; Luine, 1997; Packard and Teather, 1997; Sherwin, 1997). Estrogens can also act to alter certain pathologies such as epileptic seizure activity (Terasawa and Timiras, 1968; Saberi et al., 2001) and perhaps Alzheimer's disease (Henderson et al., 1996) and Parkinson's disease (Leranth et al., 2000). The mechanism and sites of action for the effects of estradiol on cognitive performance and epileptic seizure activity have not been established, but one probable site is the hippocampus, a sexually dimorphic, steroid-responsive brain region (Juraska et al., 1989; Roof and Havens, 1992; Woolley and McEwen, 1992).

In the adult, it has been demonstrated that the hippocampus displays a robust response to estrogen treatment and to fluctuations in ovarian hormones across the estrous cycle (Gould et al., 1990; Woolley and McEwen, 1992). Sex differences are seen in the CA1 and CA3 regions where males and females differ in dendritic branching patterns and dendritic spine densities (Gould et al., 1990) and synapses (Woolley and McEwen, 1992). When ovariectomized rats are treated with estradiol, an increase in spine density occurs

on the apical dendrites of the pyramidal cells in the CA1 region (Woolley and McEwen, 1993). Furthermore, the existence of low levels of estrogen receptors has been shown in the pyramidal cell layer of Ammon's horn, dentate gyrus, and subiculum of the adult rat (Pfaff and Keiner, 1973; Rainbow et al., 1982; Loy et al., 1988).

The developing brain is also affected by exposure to gonadal steroids resulting in sexual differentiation of the brain, which is characterized by sex-specific anatomical and physiological features of the adult animal (Harris and Levine, 1962). Ovarian steroids act during a perinatal sensitive period of development to alter the patterns of neuronal cell death, neuronal cytoarchitecture and synaptic connectivity of the hypothalamus (McEwen, 1983). Gender-related differences in hippocampal dendritic plasticity of the Ammon's horn (Juraska et al., 1989; Isgor and Sengelaub, 1998) and dentate gyrus (Juraska et al., 1985) during development have been reported. These early influences of steroids on the brain are essentially permanent and in part determine the steroid responsiveness of the adult brain (MacLusky and Naftolin, 1981). Consistent with this, estrogen receptor binding and aromatase enzyme levels have been reported to be elevated during this developmental period (O'Keefe and Handa, 1990; MacLusky et al., 1994). However, the anatomical distribution of ER's in the neonatal hippocampus has not been reported.

Until recently, the effects of estrogen were thought to be mediated by a single estrogen receptor. The cloning of ERβ in 1996 (Kuiper et al., 1996) has suggested new complexities in estrogen signaling. While both subtypes are present in the adult hippocampus (Li et al., 1997b; Shughrue et al., 1997) it is unclear which receptor is

involved in modifying hippocampal morphology and physiology in development. Early studies have demonstrated a transient elevation of ER mRNA and estrogen binding in the neonatal rat hippocampus (O'Keefe and Handa, 1990; O'Keefe et al., 1995). However, these studies were conducted before the cloning of ERβ, and do not necessarily discriminate between ERα and β isoforms or provide a careful examination of the anatomical distribution of each isoform. To further elucidate the developmental profile of estrogen receptors in the brain, we examined the distribution of ERα containing cells in the hippocampus of the male and female rat. We have demonstrated the presence of ERα in regions CA1 and CA3 of the developing rat hippocampus using immunocytochemistry and Western blot analysis, and have shown that they are found predominantly in pyramidal neurons.

## *Materials and Methods*

### Animals

Neonatal male and female rats of various ages from timed-pregnant Sprague-Dawley dams and female adult rats were used in these studies (Charles River Laboratories, Wilmington, MA). Animals were housed under a 12/12 h light dark cycle (lights on at 07.00 h) with food and water available *ad libitum*. Upon parturition, litters were sexed and thinned to 8 pups (4 males and 4 females). Animals of both sexes were sacrificed at postnatal (P) days 0, 4, 7, 10 and 15 (day of birth = P0) and females at 2-4 months

(adults). All animal protocols were previously approved by the Animal Care and Use Committee at Colorado State University.

ERα Immunocytochemistry

Neonatal rat pups were anesthetized with Halothane and sacrificed by perfusion with 10-20mL of 0.01M ice-cold phosphate buffered saline (PBS) followed by 10-20mL of freshly prepared ice-cold 4% paraformaldehyde in 0.01M PBS. Adult animals were anesthetized and sacrificed similarly, but were perfused with approximately 200mL of 0.01M PBS and 200mL 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight at 4ºC and subsequently removed and placed into 30% sucrose in 0.01M PBS at 4°C until permeated. Brains were sectioned 30μm thick on a cryostat and placed into 0.01M PBS with 0.1% Triton X-100 (PBS-TX) and 0.1% Sodium Azide. The tissue was processed for immunocytochemistry (ICC) as described previously (Kerr et al., 1995). Briefly, the tissue was first incubated with 0.3% $H_2O_2$ in 0.01M PBS to quench any nonspecific reaction with endogenous peroxidases. Subsequently, non-specific antibody binding was blocked by incubation with 4% normal goat serum (NGS) in 0.01 M PBS. Tissue was then incubated for 48 hours at 4ºC with ERα antiserum (C1355, 1:10,000; M. Shupnik, U. of VA) in the presence of 2% NGS. This antibody was raised against the 14 most carboxyl-terminal amino acids of the rat ERα, and has been described previously (Friend et al., 1997). Following primary antibody incubation, the tissue was washed (3 x 15 min. in 0.01M PBS-TX at room

temperature) and incubated for 2 hours at room temperature with a biotinylated goat anti-rabbit IgG (1:500; Vector Labs, Burlingame, CA). Tissue was washed as before and incubated with an avidin-biotin-horseradish peroxidase complex (1:500; Vector Labs) for 1 hour. Staining was visualized with a tris-buffered saline solution containing diaminobenzadine (DAB, 0.5mg/ml; Sigma) and hydrogen peroxide (0.01%) for 6-10 minutes. After developing, the tissue was rinsed and mounted on glass slides. The mounted sections were allowed to air dry overnight at room temperature and were subsequently dehydrated in graded ethanols, cleared in xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ) for analysis.

To determine immunoreactive specificity, we pre-adsorbed the antibody with the immunoreactive peptide, which was the same amino acid sequence the antibody was raised against. The antibody was incubated with 0.5ng/ml of the peptide in 0.01M PBS overnight at 4ºC. Tissue was then incubated with either a normal antibody solution, or an antibody + peptide solution for 48 hours at 4ºC. The tissue was then processed as described above for immunocytochemistry.

Double-label immunocytochemistry

For the co-localization studies, tissue from three P10 females was first processed for ERα immunoreactivity which was visualized with nickel intensified DAB. Briefly, 0.3 mg/mL of nickel ammonium sulfate was added to the DAB solution resulting in a dark blue stain. Subsequently, the tissue was washed in 0.01M PBS (3 x 15 min. at room

temperature). The tissue was then processed for γ-amino butyric acid (GABA) immunoreactivity using anti GABA serum (1:10,000, Sigma, St. Louis, MO). Essentially, the tissue was processed as above, except that after incubating with 0.3% $H_2O_2$ the tissue was blocked for 2 hours in 6% NGS. Tissue was then washed (3 x 15 min. in 0.01M PBS-TX) before the secondary antibody incubation as described above. Following a final washing step as before, this reaction was developed with normal DAB (0.5mg/mL + 0.01% $H_2O_2$) to produce a brown reaction product. After developing, the tissue was rinsed in 0.01M PBS and mounted on glass slides. The mounted sections were air-dried overnight at room temperature and subsequently the slides were processed through a series of increasing alcohols, cleared with xylene, and coverslipped with Permount (Fisher Scientific). Double-labeled cells were visualized by microscopy as cells containing a dark blue nucleus and a brown cytoplasm. The percentage of double-labeled cells was determined by taking a ratio of the number of $ER\alpha^+/GABA^+$ cells to the number of $ER\alpha^+/GABA^-$ cells in four separate coronal sections from each animal which spanned the hippocampus from dorsal to ventral. Cell counts were made using a 40X objective in three randomly chosen areas of each hippocampal subfield. Cells with a dark blue nucleus and a brown cytoplasm were considered double labeled, whereas those cells which did not contain a brown cytoplasm were considered ERα positive only.

Western Blot Analysis

Male and female animals were sacrificed at various ages and the brains were quickly removed. The hippocampi were dissected on ice and individually homogenized in 200µl of 50mM Tris buffer (pH 7.2, 4ºC) containing 1mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM leupeptin, 1 µg/ml antipain, and 1 µg/ml aprotinin. Homogenates were then centrifuged (105,000 x g) for 30 minutes at 4ºC in a Beckman L765 Ultracentrifuge and the membrane and cytosolic fractions were collected. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Protein samples were mixed 1:1 with sample buffer containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.05% bromphenol blue and boiled for 5 minutes. The samples (100µg/hippocampus, 60µg/pituitary) were separated by SDS polyacrylamide gel electrophoresis along with biotinylated molecular weight standards (Bio-Rad, Hercules, CA). The stacking gel contained 4% acrylamide while the resolving gel contained 12% acrylamide. Following electrophoresis the proteins were electrically transferred to nitrocellulose in 49.6 mM Tris, 384 mM glycine, 0.01% SDS at 30V overnight followed by 80V for 1 hour at 4ºC. The gels then were stained with Coomassie blue to confirm equal protein loading per lane. After transfer, blots were incubated in tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% non-fat milk, 2% bovine serum albumin, and 0.1% sodium azide. Subsequently, blots were incubated with ERα antiserum (C1355, 1:10,000) for 48 hours at 4ºC in TBST containing 2% non-fat milk and 0.1% sodium azide. To serve as controls, blots were also processed without

primary antibodies or with antibodies pre-adsorbed with the immunoreactive peptide. Following primary antibody incubation, the blots were washed (three times, 15 minutes each in TBST at 25ºC) and incubated with horseradish peroxidase conjugated goat anti-rabbit antibodies (1:10,000, Vector Labs, Burlingame, CA). Immunoreactive bands were visualized with an enhanced chemiluminescence system (ECL, Amersham, Arlington Heights, IL) according to manufacturers directions. To ensure that each lane was loaded with an equivalent amount of protein, the blots were stripped with 0.2M NaOH and re-probed with anti-actin serum (1:10,000; Chemicon, Temecula, CA) as described above. Following immunoblotting, digitized images of immunoreactive bands for target (ERα) and control (actin) products were imported into NIH Image software (v. 1.62) and the average OD of each band was measured (based on a grayscale of 0-256 arbitrary units). Additional background measurements were taken from each film and subtracted from these values. A ratio of ERα/actin was then determined and these values were compared across development for statistical significance using a one-way analysis of variance (StatView, SAS Institute Inc.).

## *Results*

### ERα immunoreactivity in the developing rat hippocampus

The developmental profile of ERα in regions CA1 and CA3 of the rat hippocampus as determined by immunocytochemistry are shown in Fig. 13. ERα immunoreactivity (ERα-ir) is concentrated to the nucleus of cells in the pyramidal layer

of these subregions, although a few cells throughout stratum oriens and radiatum were immunopositive as well. Immunoreactivity was consistently highest in the CA3 subregion, where not only the number of ERα-ir cells was the greatest, but the number of darkly stained cells as well. Lower levels of immunoreactivity (number of cells and darkly stained cells) were observed in CA1 with very few cells stained in the hilus and dentate gyrus. In CA1 and CA3, ERα-ir increased from postnatal (P) day 0 to P10, at which age the largest number of immunoreactive positive cells were observed. Moreover, at P10, the number of darkly stained cells was also the greatest. These levels subsequently declined, so that by P15, levels approximated those of the adult.

At early stages in development (P0-P4) no obvious anterior to posterior differences were observed in ERα-ir. However, at later stages (P7-P10) less staining was generally observed in both CA1 and CA3 of the extreme anterior pole of the hippocampus (corresponding to -2.1 mm from bregma, adult coordinate) and increased in more caudal sections (corresponding to -3.8 from bregma, adult; Fig.14).

*Figure 13 Legend: Ontogeny of ERα immunoreactivity in pyramidal cells of the rat hippocampus.* Coronal (30μm) sections were taken from postnatal and adult female rat brains and immunostained with a polyclonal ERα antiserum followed by nickel intensified peroxidase-DAB for detection. Staining is primarily concentrated to the nucleus of pyramidal cells in regions CA1 and CA3 (arrowheads) although some immunoreactive cells can be seen in stratum radiatum (arrows). Little to no immunoreactivity is observed on postnatal day (P) 0. Staining subsequently increases from P4 to P7 and reaches the highest intensity by P10. ERα immunoreactivity then declines to levels seen in the adult. Immunostaining in the hippocampus is compared to that of the preoptic area, a brain region known to express high levels of ERα. Staining in the preoptic area is intense at P0, and remains strong throughout adulthood. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

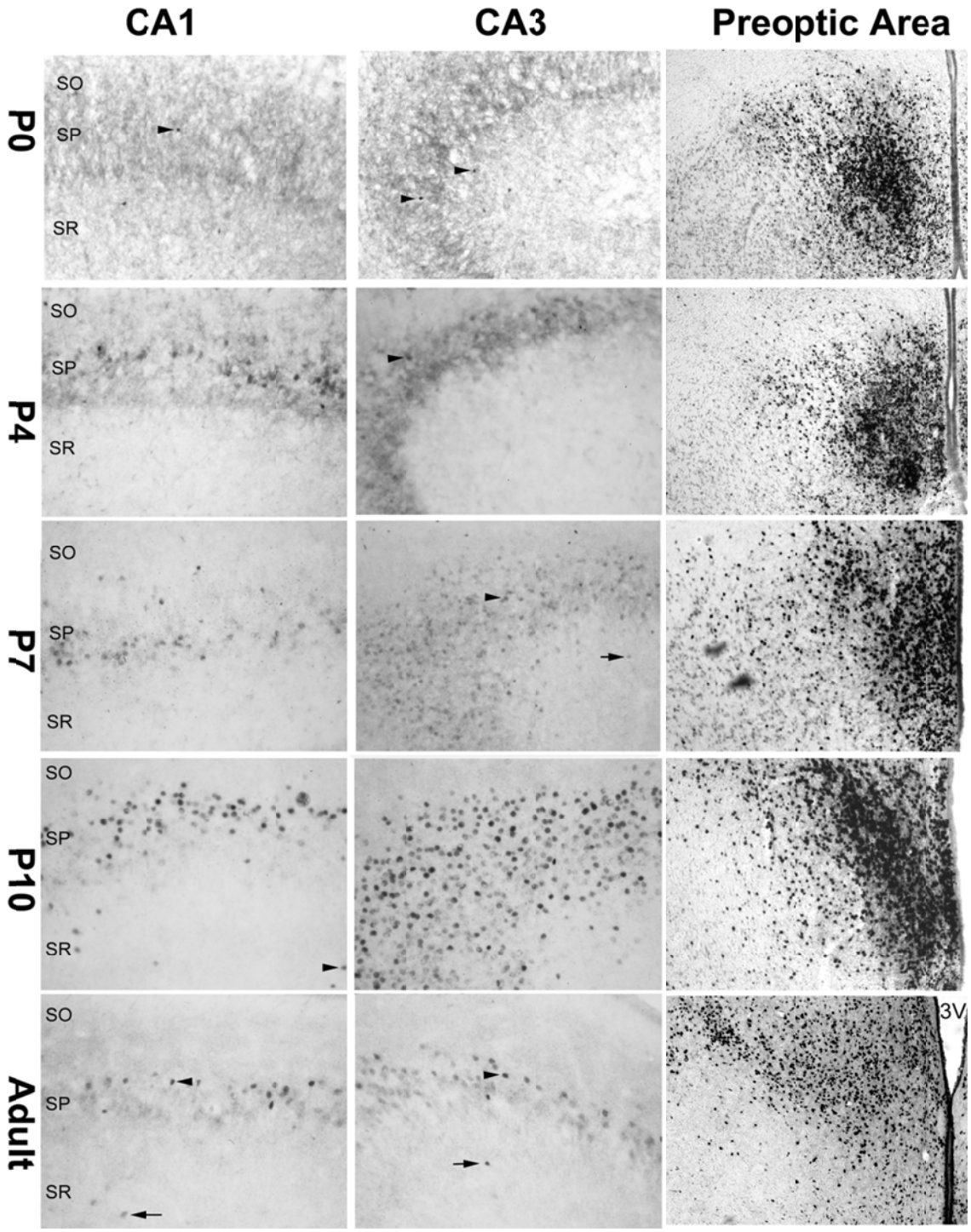



*Figure 13*

Immunoreactivity remained relatively constant from this point caudally to the posterior pole (corresponding to –5.6 from bregma, adult), where the level number of ERα-ir cells was the highest. In the adult, the highest concentration of immunoreactive cells were located –4.3 to –5.6 from bregma. Additionally, in the adult, a relatively large number of ERα-ir cells were located in the ventral hippocampus. The ventral hippocampus of the neonate, in contrast, is less defined, making it difficult to make comparisons.

Similar to earlier developmental stages, we observed a few ERα-ir cells scattered in the stratum radiatum and oriens of the adult as well. There was no significant difference in the level of ERα immunoreactivity between males and females at any age examined, where the number of immunopositive neurons appeared the same. A similar ontogenetic profile was observed in the neocortex as has been previously demonstrated (Yokosuka et al., 1995). Additionally, we compared immunostaining in the hippocampus to that of the preoptic area, a brain region known to express high levels of ERα. While ERα immunoreactivity is attenuated with maturation in the hippocampus, in the preoptic area, ERα-ir was strong at P0 and remained relatively high through adulthood (Fig. 13).

To determine if the staining we observed was specific for ERα, we pre-adsorbed the antibody with the immunoreactive peptide. The peptide was made corresponding to

Figure 14 Legend: Schematic drawings representing the immunohistochemical distribution of ERα in three coronal sections of the postnatal day 10 rat brain. Relative numbers of immunoreactive cells are represented by black circles; where the highest numbers of cells are represented by the largest circles, intermediate numbers are represented by medium sized circles, and the lowest numbers are represented by the smallest circles. CA1-3, fields CA1-3 of Ammon's horn; cg, cingulum; DG, dentate gyrus; Ent, entorhinal cortex; ic, internal capsule; LV, lateral ventricle; ml, medial lemniscus; S, subiculum. Drawings modified from the atlas of (Paxinos and Watson, 1986).

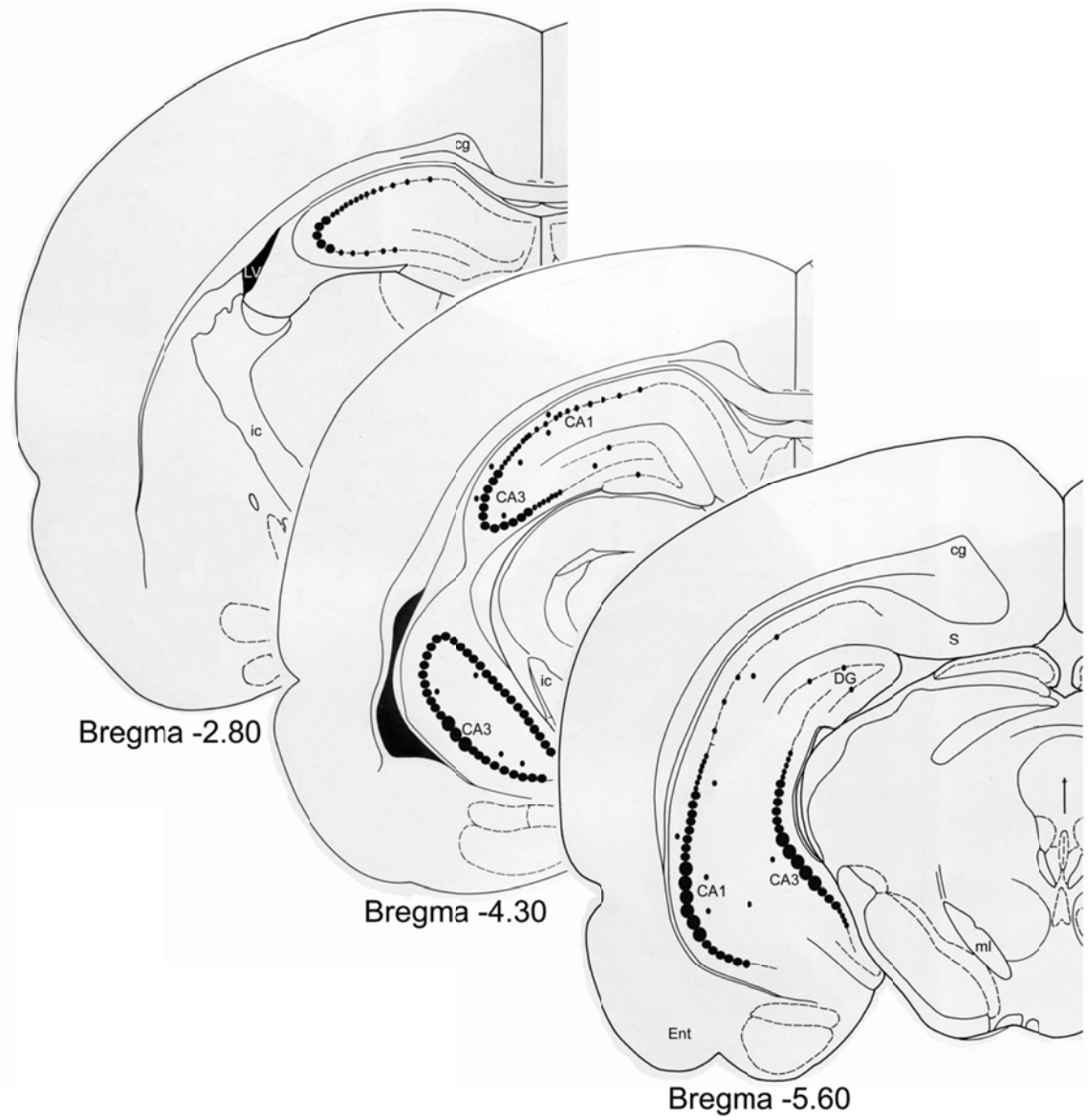

cg
LV
ic
CA1
CA3
Bregma -2.80
CA3
ic
cg
S
DG
CA1
CA3
ml
Ent
Bregma -4.30
Bregma -5.60

*Figure 14*

the last 14 amino acids of ERα. Pre-incubation of the antibody with an 0.5ng/mL of the immunoreactive peptide completely abolished all staining in the hippocampus and other ERα immunoreactive brain regions, such as the preoptic area (data not shown).

Western blot analysis

Using Western blot analysis, we determined that the ERα expressed in the developing male and female rat hippocampus is a full-length protein (Fig. 15A). The antibody used in this experiment recognized a protein of approximately 67 kDa, which is the size for the full length ERα as previously reported (Furlow et al., 1990). Initial experiments were conducted to investigate the presence of ERα in both membrane and cytosolic fractions following centrifugation. These experiments demonstrated that nearly all ERα was recovered in the cytosolic fraction (Fig. 15A), and no immunoreactive bands were detectable in the membrane fraction (not shown).

The protein recognized in hippocampus correlates with a protein of the same size in the pituitary (Fig. 15A), a tissue known to express high levels of ER. We were also able to detect an intense band of the same size in the hypothalamus and uterus (data not shown). In addition, the ontogenetic expression profile for ERα by Western blot closely matched that of ERα-ir in the hippocampus. That is, immunoreactivity levels increased from P0 to P10, where the level of expression is higher than all other ages examined ($p<0.005$), with a subsequent decline to levels observed in the adult (Fig 15B).

Figure 15 Legend: Western blot analysis of ERα protein in the developing rat hippocampus. (A) A single band of approximately 67 kDa detected by Western blotting of cytosolic protein from the hippocampus of various days during postnatal (P) development. Relative levels of hippocampal cytosolic protein (100μg) are compared to that of adult pituitary cytosolic protein (60μg), which expresses abundant levels of ERα. The protein detected in the hippocampus is of the same molecular weight as full length ERα, which is the predominant form found in the pituitary. (B) Following Western blot analysis of ERα and actin in the developing hippocampus, mean optical densities were determined of immunoreactive bands. Background levels were subtracted from these measurements and a ratio of ERα/actin was determined to give a net mean density for each age. Each bar represents the mean ± S.E.M. of 3 independent experiments. (*) Significantly higher than all other ages examined ($p < 0.005$).

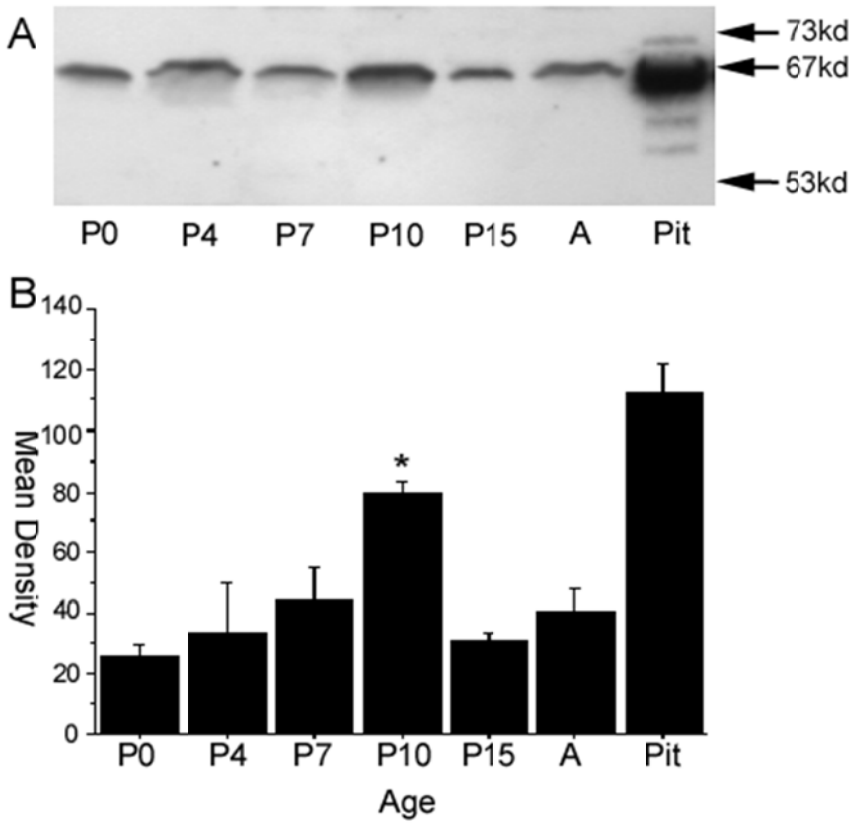

A
73kd
67kd
53kd
P0
P4
P7
P10
P15
A
Pit
B
Mean Density
140
120
100
80
60
40
20
0
*
P0
P4
P7
P10
P15
A
Pit
Age

*Figure 15*

When immunoblots from males and females were compared, it was determined that there was no significant difference in the level of ERα expression between the sexes at any age examined. Furthermore, pre-adsorption of the antibody with the immunoreactive peptide completely eliminated the 67 kDa band (data not shown).

ERα/GABA double label immunocytochemistry

To assess whether ERα is localized to pyramidal cells or interneurons of the developing rat hippocampus, we used double label immunocytochemistry for ERα and GABA, a marker of interneurons. Postnatal day 10 females were used in these studies because the level of ERα immunoreactivity is highest at this age. We have demonstrated that while ERα and GABA are co-localized in a few cells of the neonatal hippocampus, the majority of ERα-ir neurons are GABA negative (Fig. 16). This is especially the case in the CA3 subregion, where 96% ± 0.8% of ERα-ir cells are *not* GABA-ir (92% ± 2.1% in CA1), implying that they are pyramidal cells, rather than GABAergic interneurons.

Figure 16 Legend: Double label immunocytochemistry of ERα and GABA in the pyramidal cell layer of the P10 rat hippocampus. Cells in the CA1 region of a P10 (A, B) and adult (C-E) rat hippocampus were immunostained for ERα and GABA. Cells immunoreactive (ir) for ERα contain a dark blue nucleus, while cells which are immunoreactive for GABA contain a brown cytoplasm. Low power (A) and high power (B) photomicrographs of the CA1 region of a P10 female hippocampus demonstrating ERα and GABA immunoreactivity. Cells in stratum pyramidale tend to be either ERα-ir (black arrowheads) *or* GABA-ir (white arrows) but not both. Some ERα-ir cells can be seen in stratum radiatum as well (black arrows). Less ERα-ir is observed in the CA1 region (C) of the adult compared to the CA1 region of the P10 animal (A). A few scattered ERα-ir cells can be seen in the granule cell layer (D; black arrowheads) and stratum radiatum (E; black arrows) of the adult as well. GABA-ir cells can also been seen in these subregions, but these are not ERα-ir. SO; stratum oriens, SP; stratum pyramidale, SR; stratum radiatum.

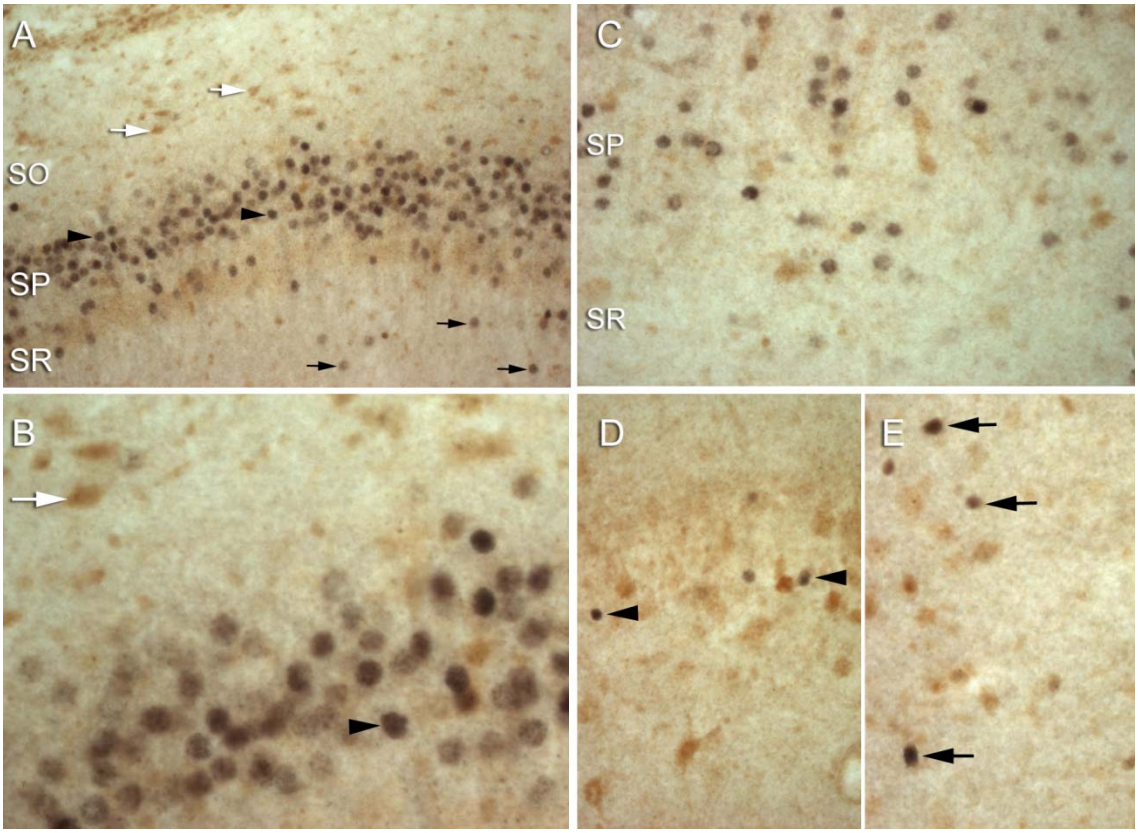



*Figure 16*

## *Discussion*

In the present study, using a specific antibody that detects full length ERα, we examined the ontogeny and distribution of ERα containing cells in the developing male and female rat hippocampus. Our results demonstrate that the ERα phenotype is transiently expressed in the developing rat hippocampus. At postnatal day 0, there is very little expression of ERα in the rat hippocampus. ERα can be detected by P4 and reaches its maximum levels by P10. ERα-ir then decreases, so that by P15 the levels observed approximate those of the adult. Moreover, expression of ERα is concentrated to the nucleus of pyramidal cells in the CA1 and CA3 regions of the developing rat hippocampus, although some immunoreactive interneurons were also detected.

Our results also show a distribution pattern that is consistent with previous reports of ER mRNA levels, where within the pyramidal cell layer, the highest density is found in the CA3 region, intermediate levels are found in the CA1, and lower levels are found in the dentate gyrus (O'Keefe and Handa, 1990). However, the ontogenetic pattern of expression that we observed differs somewhat from that previously reported using RNase protection assay (O'Keefe et al., 1995) and receptor binding techniques (O'Keefe and Handa, 1990). In those studies, the peak of ERs in the neonatal hippocampus was approximately postnatal day 7, while the peak we have observed for ERα is on postnatal day 10. The earlier studies were conducted prior to the identification of ERβ though, and

did not differentiate between the two receptor isoforms. The presence of ERβ could create the discrepancy between those studies and this one.

A recent report has demonstrated the presence of ERβ mRNA in the neonatal mouse hippocampus (Ivanova and Beyer, 2000). In this report it was demonstrated using semi-quantitative RT-PCR that male mice have transiently higher levels of ERα and β mRNA than females at P7. While previous work in our laboratory has shown a similar ontogenetic profile for ER message levels in the hippocampus using *in situ* hybridization (O'Keefe et al., 1995), we have not observed a sex difference in either ERα mRNA (O'Keefe et al., 1995) or protein levels (current study and O'Keefe and Handa, 1990). The use of different species (mouse vs. rat) in these studies could explain this discrepancy. Furthermore, Ivanova and Beyer (Ivanova and Beyer, 2000) did not examine levels of ERβ protein and it is possible that variations in either ER message stability or degradation could account for the differences they report. Finally, we have observed developmental differences in hippocampal subregions (CA1 vs. CA3) that would have been missed if the entire hippocampus, including the dentate gyrus and subiculum were homogenized together. In these brain regions we have observed very little ERα staining.

The data presented in this study also raise another interesting question concerning the time frame for organizational effects of steroid hormones in different brain regions. For instance, extensive studies examining the hypothalamus have demonstrated that in rats, gonadal hormones influence sexual differentiation of the hypothalamus from

gestational day 18 through about the second week after birth (MacLusky and Naftolin, 1981), and these effects are likely mediated through the estrogen receptor. Our results, showing the peak of ERα expression at P10 in the hippocampus, which is relatively late in relationship to the "critical period" of the hypothalamus, could suggest that the hippocampus possesses a critical period that is later or prolonged compared to that for the hypothalamus. This is consistent with the fact that the hippocampus finishes developing at a later age than the hypothalamus (Bayer, 1980; Jacobson and Gorski, 1981a; Seress, 1985), and studies in the hypothalamus have shown that different critical periods exist for different functions (Handa et al., 1985). Alternatively, it may be that the transient expression of ERα in the hippocampus is not related to sexual differentiation at all.

Based on the morphology and localization of immunoreactive cells, it appears that ERα is localized predominantly to hippocampal pyramidal neurons, rather than non-spiny interneurons. This is consistent with data demonstrating the localization of ERα messenger RNA in the developing hippocampus using in situ hybridization (O'Keefe et al., 1995). However, early reports suggested that, in the adult, ERs are concentrated to interneurons rather than pyramidal cells (Weiland et al., 1997), even though most morphological and physiological effects of estrogen have been demonstrated in pyramidal cells (Woolley and McEwen, 1992; Lavenex et al., 2000). For instance, it has been shown that following treatment with estradiol, it is primarily pyramidal cells that become activated when using c-Fos as an indicator (Rudick and Woolley, 2000). Our study, in contrast to earlier reports, further demonstrates that the ERα-ir cells are

pyramidal neurons, although the staining in the adult is much lighter than earlier periods in development. While we detected a few scattered ERα-ir cells in stratum radiatum and oriens, nearly all immunoreactivity was observed in stratum pyramidale, supporting our conclusion that the ER-ir cells are pyramidal neurons. Moreover, it is unlikely that the ERα-ir cells are interneurons based on their number, considering that interneurons constitute a relatively small percentage of all neurons in the hippocampus (Olbrich and Braak, 1985). A recent report supports the suggestion that ERα is only localized to non-spiny interneurons in the developing hippocampus (Orikasa et al., 2000). However, the earliest age examined in that study was postnatal day 14, a time point where we have not observed any difference from adults, and the levels of ERα expression are relatively low. Additionally, the report of Orikasa et al. (2000) used *in situ* hybridization histochemistry with digoxigenin-labeled probes to assess mRNA levels and a different ERα antiserum. Therefore, while the data from Orikasa et al. (2000) are not inconsistent with the results presented here, differences in methodologies could explain the discrepancies. Additional evidence that ERα is primarily localized to pyramidal cells comes from our co-localization studies where, in the developing rat hippocampus, very few ERα-ir cells are also immunoreactive for GABA, thus, strongly suggesting that the ERα-ir cells are pyramidal cells rather than interneurons. Unfortunately, no specific marker of all pyramidal cells exists to our knowledge, and thus we are unable to make an absolute determination of ERα-ir neuron type.

While the number of immunoreactive cells in the adult was less than that observed at P10, the number of ERα positive cells visualized was much greater than originally reported (Weiland et al., 1997). The distribution we observed in the adult more closely resembled the recent report of Shughrue and Merchenthaler (Shughrue and Merchenthaler, 2000), where pyramidal cells of regions CA1-3 were highly labeled, especially in the ventral hippocampus. The report by Shughrue and Merchenthaler (Shughrue and Merchenthaler, 2000) provides additional anatomical evidence of the presence of ERs in pyramidal cells of the adult hippocampus, supporting our results in the developing and adult animal as well. Similar to our findings, this article has challenged the notion that ERs are only found in non-spiny interneurons as was originally reported. Our observations, and those of Shughrue and Merchenthaler (Shughrue and Merchenthaler, 2000), fit with the majority of physiological data showing direct effects of estrogen on pyramidal cells.

The recent discovery of ERβ raised the possibility that many of the effects of estrogen could be mediated by this receptor. While messenger RNAs for ERβ, and to a lesser extent ERα, have been detected in the adult hippocampus (Simerly et al., 1990; Shughrue et al., 1997), it has not been demonstrated whether ERβ protein exists or not. Furthermore, all of the previous estrogen binding studies have either been conducted in the adult or do not discriminate between the two receptor types. Our data suggest that the receptor responsible for mediating estrogens actions in the hippocampus is ERα, but does not rule out a similar role for ERβ. Considering the important role that ERβ potentially

plays in the hippocampus, studies examining the anatomical distribution of both message and protein for this receptor during development should to be conducted. Such data are currently missing from the literature and would provide additional insight into the complexity of ER signaling mechanisms.

The identification of multiple splice variants of ERβ mRNA (Petersen et al., 1998) in the normal rat brain has suggested an additional level of complexity in estrogen signaling. Recently, it has been suggested that the major splice variant of ERβ in adult hippocampus is one that lacks the fourth exon (Price et al., 2000). This δ4 variant does not bind estrogen. Similarly, splice variants of ERα have been identified (Pfeffer et al., 1996; Pasqualini et al., 1999). An ERα variant mRNA, lacking exon four, has been isolated from the adult rat brain (Skipper et al., 1993), but does not appear to be present in hippocampus (Price et al., 2000). Most of the ERα variant mRNAs have only been isolated in cancer tissues, cell lines or non-brain tissues. In this study we have demonstrated that the ERα protein detected in the hippocampus is consistent with the size for the functional full-length ERα subtype previously described (Furlow et al., 1990).

During normal development, the male brain is exposed to high levels of gonadal steroids during a perinatal sensitive period. These steroids act to organize the brain in a male direction resulting in permanent morphological and physiological changes. It has been proposed that the organizational effects of estrogen seen early in development create a hormonally responsive brain in adulthood (Phoenix et al., 1959). The transient expression of ERα in the rat brain implicates it in mediating the effects of estrogen

during development. Furthermore, during the second postnatal week, a time when we observe the highest concentration of ERα, the plasma levels of estradiol are also high (Meijs-Roelofs et al., 1973; Dohler and Wuttke, 1975). It follows then, that estrogen acting on the ERα receptor in the hippocampus around the tenth postnatal day of development could be responsible for creating a hippocampus that has an altered responsiveness to gonadal hormones in adulthood. For instance, although castration of male rats reduces spine density in adulthood, estrogen treatment of adult gonadectomized males has no effect on spine density in contrast to the effect of estrogen in females (Lewis et al., 1995). It is also possible that estrogen is acting during the neonatal period to sculpt the morphology or connectivity of the hippocampus in a male (or female) direction and to function differently in adulthood, regardless of the adult steroid environment. Unfortunately, most studies of the effects of estrogen in the hippocampus have been conducted in the adult, and have overlooked the neonatal period when the organizational effects of steroid hormones occur.

The classical role of ER in neurobiology is to regulate the expression of specific target genes by binding DNA directly through estrogen response elements (Beato et al., 1989). Previous work in this laboratory has shown that in the neonatal hippocampus, ER can bind DNA (O'Keefe and Handa, 1990). In this study, we have demonstrated a transient increase of ERα immunoreactivity in the nucleus of pyramidal cells of the developing rat hippocampus. The fact that ERα immunoreactivity is concentrated to the nucleus is consistent with a genomic form of action that has been extensively described.

Until recently, based on studies in adult rat brain, it was believed that ERs only existed in non-pyramidal neurons. This created speculations that estrogen was acting though sparse interneurons or non-genomic mechanisms to alter hippocampal morphology. Based on our data, it appears that in the neonate, the effects of estrogen to developmentally organize the hippocampus are mediated directly through hippocampal pyramidal cells, rather than GABAergic interneurons. It is also possible that activation of ERα in development results in transcriptional changes that alter the morphology and physiology of developing hippocampal pyramidal neurons. These findings may contribute to our understanding of the cellular mechanisms by which estrogen modulates hippocampal related behaviors.

# CHAPTER 4

## EXPRESSION OF ESTROGEN RECEPTOR BETA (ERβ) mRNA SPLICE VARIANTS IN THE DEVELOPING RAT HIPPOCAMPUS


### *Abstract*

During development, estrogen has been shown to have a variety of effects on hippocampal neurons. In the brain, the actions of estrogen are mediated by two different estrogen receptors (ER); α and β. ERβ mRNA has been has been shown to be variably spliced in brain tissue, resulting in proteins with different functional properties. Wild type ERβ has been designated β1, an insert between exon 5 and 6 in the ligand binding domain (LBD) has been termed β2, and a deletion of exon 3 or 4 has been termed δ3 or δ4 respectively. In this study, we examined the expression and regulation of ERβ2 mRNA splice variants (with and without δ3 together) in the hippocampus, hypothalamus and cortex throughout early postnatal development using quantitative real-time RT-PCR. These results were compared to data obtained using conventional PCR methods. Additionally, because β2 variants have been reported to have a lower affinity for estradiol than β1, we used an estrogen receptor binding assay to assess whether the pattern of estrogen binding during development was consistent with the mRNA isoforms expressed at the same period. Our results suggest a development change in the levels of ERβ2

mRNA isoforms. From postnatal day (P) 0-4, the β2 variants are expressed at very low levels in the hippocampus. Based on the changes in amino acid structure, it is likely that this isoform does not contain a functional LBD. At P7 a significant change in the pattern of ERβ splice variants occurs in the hippocampus such that the β2 variants are expressed significantly higher. Conventional PCR studies suggest wild type and δ4 variants are also present in the developing hippocampus. Beginning on postnatal day 15 and continuing through adulthood, there is a gradual decrease in the level of expression for the ERβ2 splice variants. In contrast to the hippocampus, in the prefrontal cortex and hypothalamus ERβ2 variants do not change significantly over development. Conventional PCR methods suggest that the wildtype form is predominantly expressed in these brain regions during development. Next, to determine if ERβ splicing might be hormone regulated during development, we gonadectomized male rat pups at P0 and subsequently treated them with a single injection of 10μg of estradiol benzoate or oil vehicle. Gonadectomy caused a change in the expression pattern of ERβ mRNA isoforms, where ERβ1 was expressed at the highest levels at early ages. Estrogen treatment results in a pattern of expression similar to that of intact males. These data suggest that different ERβ mRNA isoforms are present during development, including ERβ2 which has an 18 amino acid insertion in the ligand binding domain.

## *Introduction*

Estrogen signal transduction is mediated by two estrogen receptors, α and β, (see Katzenellenbogen, 1996) which are encoded by different genes. These receptors are members of the nuclear receptor family of transcription factors (Tsai and O'Malley, 1994). All members of the nuclear receptor superfamily share characteristic structural and functional domains able to act relatively independently of each other. These domains include the ligand-binding domain located at the C-terminal half of the protein, the DNA-binding domain located centrally, and a variable transactivation domain located at the N-terminal end (Evans, 1988).

Additionally, for ERα and ERβ, a number of messenger RNA variants have been described (Fuqua et al., 1993; Pfeffer et al., 1996) which are generally the result of an imprecise pattern of mRNA splicing that gives rise to a family of correctly processed and exon-skipped transcripts that disrupt the normal domain structure of the receptor. While most of the ERα splice variants have been described in cancer cell lines or tumors, a few reports have demonstrated their presence in normal tissues such as the pituitary (Friend et al., 1997; Fasco et al., 2000; Pasqualini et al., 2001). Several ERβ mRNA splice variants have also been described, many of which are endogenously expressed at relatively high levels in normal tissues (Petersen et al., 1998; Raab et al., 1999; Inoue et al., 2000; Price and Handa, 2000; Price et al., 2000).

There are currently at least five ERβ mRNA variants that have been described to date including the wildtype form which has been designated ERβ1. ERβ2 has an in-

frame insertion of 54 nucleotides between exon 5 and 6 that composes 18 amino acids within the ligand-binding domain (Petersen et al., 1998). As predicted, this hormone binds estrogen with a much lower affinity than the wildtype receptor and requires 100-1000 times more estrogen to fully activate transcription (Petersen et al., 1998; Hanstein et al., 1999). The δ3 variant encodes an isoform resulting from an in-frame loss of 117 base pairs in the DNA binding domain. As a result, this splice variant does not bind DNA efficiently, but rather appears to enhance transcriptional activation through an activator protein-1 (AP-1) response element in the promotor (Price et al., 2001). Lastly, a δ4 variant results from the deletion of the fourth exon encoding the nuclear localization signal (Price et al., 2000). As a result, this receptor variant appears to localize to the cytoplasm. These ERβ mRNA splice variants modify the organization of the structural domains, subsequently resulting in proteins with different cellular and subcelluar localizations and altered functional properties (Hanstein et al., 1999; Inoue et al., 2000; Price et al., 2001). For instance, it has been demonstrated using transient transfection experiments with green fluorescent protein tagged splice variants that the subnuclear distribution is highly dependent upon the ERβ splice variant expressed (Price et al., 2001).

In this study, we used conventional and quantitative real-time PCR to examine the expression of ERβ mRNA splice variants in several regions of the developing rat brain, including the hippocampus, medial basal hypothalamus, and cortex. Our results demonstrate that all ERβ variants are expressed in brain tissue, and in some cases the

expression levels of variants were equal or near wildtype levels. Additionally, the expression pattern was unique for the ERβ splice variants depending on the brain region examined. This was particularly the case for the hippocampus, which expressed high levels β2 variants during development. In contrast, the medial basal hypothalamus and cortex did not show significant changes in ERb mRNA splice variants during development.

## *Materials and Methods*

### Animals.

Timed-pregnant Sprague-Dawley females and adult male and female adult rats were obtained from Charles River Laboratories (Portage, MI). Animals were housed under a 12/12 h light dark cycle (lights on at 07.00 h) with food and water available *ad libitum*. Upon parturition, litters were sexed and thinned to 8 pups (4 males and 4 females). Shortly after birth (4-6 hours), some pups were gonadectomized under hypothermia anesthesia. Of these, half received a single subcutaneous injection of 10μg estradiol benzoate in 50μl sesame oil, while the other half received oil alone. This protocol has been shown to result in a decrease in plasma testosterone levels, an increase in estrogen receptor levels and the abolishment of male sexual behavior (Ulibarri et al., 1990; Kuhnemann et al., 1995; McCormick et al., 1998; Atanassova et al., 1999), presumably by overcoming the ability of circulating alpha-fetoprotein to sequester circulating

estrogen from the brain. Animals were subsequently sacrificed at postnatal (P) days 4, 7, 10, 15, 20 and 25 (day of birth = P0). Adult males and females were sacrificed at 3-4 months of age. An additional group of intact animals were sacrificed on P0. All animal protocols were previously approved by the Animal Care and Use Committee at Colorado State University.

Total RNA isolation and reverse transcription.

Neonatal rat pups were decapitated, and the brains quickly removed and the medial basal hypothalamus and hippocampal regions CA1 and CA3 were dissected. Total RNA was isolated according to the protocol of (Chomczynski and Sacchi, 1987). Briefly, brain tissue was homogenized in 250µl of GIT buffer (4M guanidinium isothiocyanate/25mM NaCitrate, pH 7.0/0.5%sarcosyl/0.1M β-mercaptoethanol) on ice. Subsequently, 25µl of 2M NaOAc (pH 4.0), 250µl of buffer-saturated phenol (pH 4.3) and 50µl of chloroform/isoamyl alcohol (49:1) was added and the mixture vortexed. The samples were then centrifuged at 14,000 x g for 20 minutes at 4ºC. The aqueous phase was recovered and the RNA was ethanol precipitated. The resulting RNA was washed with ice-cold 70% ethanol, and reconstituted in 50µl RNAse free water. The RNA content was measured with a spectrophotometer and only those samples with a 260/280 ratio above 1.6 were used.

2 µg of total RNA was reverse transcribed with Superscript II reverse transcriptase (RT; Gibco/BRL, Rockville, MD) using oligo-dT primers,

deoxyribonucleotides (dNTPs; 100 mM each), 1st strand buffer (100 mM Tris-Cl/900 mM KCl/1 mM $MgCl_2$) and 2.5 mM dithiothreitol (DTT). The reaction was carried out at room temperature for 10 minutes followed by 50 minutes at 42ºC. The RT was then denatured at 95ºC for 10 minutes and stored at –80ºC until used.

Real-time quantitative polymerase chain reaction (PCR).

Real-time quantitative PCR was performed using the LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN). In this system, PCR occurs in borosilicate glass capillaries that have a high surface-to-volume ratio to ensure rapid equilibration between the air and the reaction components. A highly specific double-stranded (ds) DNA binding dye, SYBR Green I (Molecular Probes, Eugene, OR), which only fluoresces when bound to dsDNA, is used to determine the concentration of amplified products. SYBR Green I binds to the minor groove of dsDNA and fluorescence is greatly enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending the amount of dsDNA that is present. The 530 nm fluorescence is recorded at the end of the elongation phase, and increasing amounts of PCR product are monitored from cycle to cycle. By comparing the amount of unknown cDNA to a curve of amounts of a given cDNA amplified concurrently, real-time PCR eliminates the need for competitive in-tube standards with identical primer sets as targets (Morrison et al., 1998).

To prevent nonspecific amplification we used hot-start PCR with dNTPs, specific primers, PCR buffer (100 mM Tris-Cl, 1.5 mM $MgCl_2$), 0.5 units Taq polymerase, 0.5

units Taq antibody (Gibco/BRL) and 2 μl of 10X stock SYBR Green I. Specific primers for ERβ2 were: Forward-GGATGCTCACTTCTGCGCCGTCT and Reverse-CTTCATCTGCGCAACGTGCCAGT from 5' to 3'. Primers were developed using OLIGO software (v. 6.51; Molecular Biology Insights, Cascade, CO). All samples were amplified at 40 cycles that is approximately five to ten cycles beyond the beginning of the linear phase of amplification. Specifically, an initial melting step was done at 95ºC for 2 minutes followed for 40 cycles of 95ºC melting step for 1s, an annealing step (60ºC) for 5s, and a 72ºC elongation step for 13s. In all experiments, samples containing no template were included to serve as negative controls. To ensure that the standard and unknown samples amplified equivalently additional control experiments were conducted in which a known amount of target cDNA (from the standard curve) was added to the unknown samples. These results were then compared to the sum of those obtained from the known and unknown samples amplified independently and were not significantly different ($p = 0.797$).

Construction of the ERβ2 standard curve.

To determine the absolute concentration of the target transcript, conventional PCR for ERβ2 was used to generate a cDNA. Conventional PCR was performed on full length ERβ2 that had been excised from an expression vector containing this splice variant and was a gift from Dr. T. Brown (Pfizer, Inc., Groton, CT). The amplified cDNA was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA)

according to the manufacture's directions. The purified PCR products were serially diluted at a range of 10ng-10fg and this curve was run in duplicate alongside the unknown samples.

[$^3$H] Estrogen Binding Assay.

Cytosols from P7 and adult hippocampi were subject to an *in vitro* binding assay to determine if there were developmental differences in the ability of ERs to bind 17β-estradiol ($E_2$). The results were compared to those from P7 hypothalamus and cortex and adult hippocampus and hypothalamus. Tissue samples were homogenized in 300µl of ice-cold TEGMD buffer (10mM Tris-Cl, 1.5mM EDTA, 10% glycerol, 25mM molybdate, and 1mM dithiothreitol, pH 7.4) in Dounce tissue grinders (Dounce Co., Vineland, NJ). The homogenates were transferred to 9 x 50mm polyethylene tubes and centrifuged at 105,000 x g for 30 minutes at 4°C in an ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA) with a fixed angle rotor to separate the extract from the nuclear and membrane fractions. Aliquots (100µl) of the supernatant cytosols were incubated with increasing amounts of [$^3$H] $E_2$ (0.01-50nM) for fours hours at room temperature in a total incubation volume of 150µl. Parallel incubations containing a 200-fold excess of Diethylstilbestrol (DES) in addition to [$^3$H] $E_2$ were used to assess nonspecific binding. After four hours, bound and unbound [$^3$H] $E_2$ were separated by passing the incubation reaction through a 1mL lipophilic Sephadex LH-20 column. The columns were re-equilibrated with 100µl TEGMD buffer and the incubation reactions were added to each column and allowed to

incubate on the column at 4°C for 30 minutes. Next, the columns were washed with 600µl TEGMD and the flow through was collected. To these samples, 4ml of scintillation fluid was added and the samples were counted for 5 minutes in a scintillation counter.

Statistical analyses.

All analyses were performed using an analysis of variance (ANOVA, StatView, SAS Institute Inc.). Significant values were subsequently verified with the Tukey-Kramer post hoc analysis.

## *Results*

### Expression of ERβ mRNA splice variants in the developing rat hippocampus

Using quantitative and conventional PCR methods we examined the expression of ERβ mRNA isoforms in the neonatal rat hippocampus during development (Fig 17). Our results using conventional PCR demonstrate that, in the hippocampus, ERβ mRNA splice variants are expressed a throughout development at different relative intensities depending on the age. Initially, using conventional PCR with primers that span all known splice variants, we were able to examine the relative distribution of each of these ERβ variants during development (Fig 17A). On postnatal day 0, the ERβ2δ3 and δ4 variants are expressed at the highest concentration in the hippocampus. On postnatal day

7, the ERβ2δ3 isoform is expressed at the highest level, with lower levels of the other variants (including wildtype and δ4) present. Notably, because the δ3 variants lack the carboxyl terminal end of the DNA binding domain, they doesn't bind estrogen response elements efficiently, but rather act to enhance transcription through an activator protein-1 (AP-1) site (Price et. al., 2001). The pattern of expression observed on postnatal days 10 and 15 are very similar to that observed on P7 with ERβ2δ3 expressed at the highest level. In adult samples, the wildtype ERβ1 form is expressed at the highest levels. Additionally, while expressed at lower levels than β1, the δ3 (both β1 and β2) and δ4 variants are detectable in adults. Due to the variability associated with these conventional RT-PCR experiments, we were unable to make definitive claims concerning changes in expression of ERβ splice variants over development. Because of this, we employed a quantitative real-time PCR approach that allowed us to examine the β2 variants (with and without the δ3 component) across development. With this approach we used specific PCR primers that were located in exon 2 and the β2 insert. With these primers we simultaneously amplified cDNA samples from hippocampal regions CA1 or CA3 alongside samples containing a known concentration of ERβ2 cDNA and these data are presented in figure 17B. These results demonstrate that a general trend of increasing expression for ERβ2 mRNA over the first two weeks of development, such that, by P7 the level of expression was significantly greater than at birth ($p < 0.02$). While this approach is unable to discriminate between the ERβ2

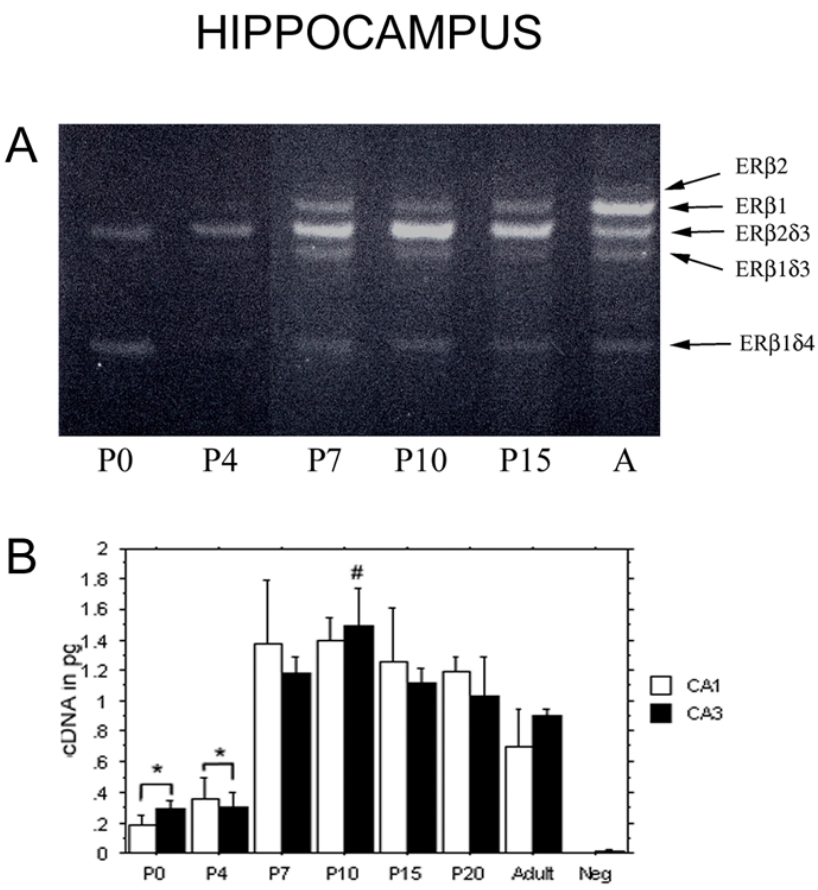



*Figure 17*

Figure 17 legend: Expression of ERβ mRNA splice variants in the developing rat hippocampus. Expression of ERβ mRNA splice variants in the hippocampus were examined using conventional (A) and quantitative real-time (B) PCR. A) At most ages examined, ERβ2δ3 was expressed at the highest levels relative to the other variants. A notable exception was the adult, where the wildtype form predominated. B) Across development, ERβ2 levels are very low from P0-4, then increase to their peak at P10 then drop slightly to levels observed in adults. (*) Significantly less than all other ages ($p < 0.02$). (#) Significant greater than adults ($p < 0.04$)

and ERβ2δ3 variants, it does provide quantitative evidence demonstrating a transient increase in ERβ2 mRNA splice variants during early hippocampal development.

Expression of ERβ2 mRNA splice variants in the hypothalamus and cortex do not change over development.

To determine if the differential expression of ERβ mRNA splice variants was brain region specific, we also examined their expression in the medial basal hypothalamus (Fig. 18) and cortex (Fig. 19) of the developing rat. These brain regions have been shown to express wildtype ERβ mRNA in adulthood (Shughrue et. al., 1997). Our results using conventional RT-PCR suggest that in adults and every age examined during development, ERβ1 was expressed at the highest levels relative to the other variants. Moreover, in each of these cases, ERβ2 was expressed at the next highest relative intensity with lower levels of δ3 and δ4 detected in the hypothalamus and hippocampus respectively. Next, using real-time PCR, we demonstrated that ERβ2 splice variants in the developing hypothalamus (Fig. 18B) and cortex (Fig 19B) do not change significantly over development. In the medial basal hypothalamus, we did observe a general trend towards decreasing levels of the β2 isoforms with increasing age. However, the decreases in expression with increasing age were not statistically significant ($p > 0.45$), and it is unclear whether the gradual decline has any physiological relevance.

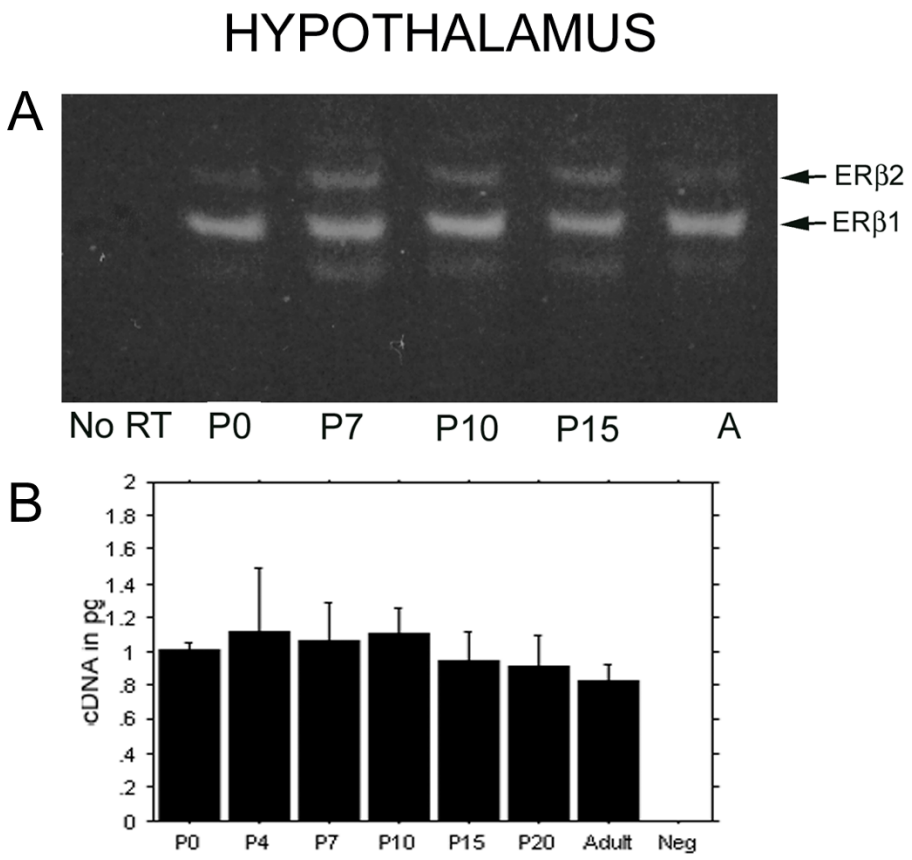



*Figure 18*

Figure 18 Legend: Expression of ERβ mRNA splice variants in the medial basal hypothalamus during development. Expression of ERβ mRNA splice variants in the hypothalamus was examined using conventional (A) and quantitative real-time (B) PCR. A) The wildtype ERβ1 isoform is predominantly expressed at all ages examined with lower levels of β2 also observed consistently. While their expression is quite variable, δ3 variants are sometimes detectable. B) Quantitation of ERβ2 variants did not reveal a significant developmental change in expression ($p > 0.85$). A trend is observed where the levels of β2 expression gradually decline with increasing age.

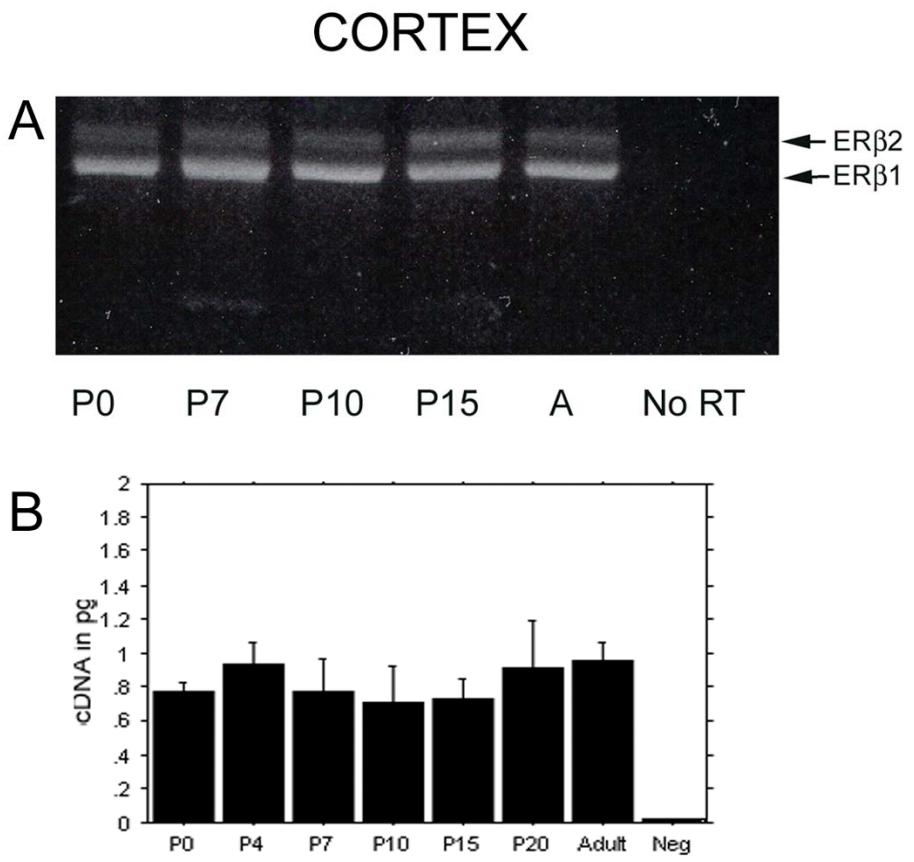



*Figure 19*

Figure 19 legend: Expression of ERβ mRNA splice variants in the cortex during development. Expression of ERβ mRNA splice variants in the cortex was examined using conventional (A) and quantitative real-time (B) PCR. A) The wildtype ERβ1 isoform is predominantly expressed at all ages examined with lower levels of ERβ2 also consistently detected. While observed only sporadically, we were able to detect δ4 variants in the cortex at early ages. B) Quantitation of ERβ2 (including β2 and β2δ3) variants did not reveal a significant change in expression over development ($p > 0.92$).

In these experiments it was not uncommon to see very low levels of δ3 variants in the hypothalamus, although the expression of these variants (ERβ1δ3 and β2δ3) was variable and inconsistent. Interestingly, unlike the hippocampus, the δ4 variant is undetectable with conventional PCR methods at all ages examined.

ERβ mRNA splice variants are regulated by estrogen in the developing hippocampus

To determine if ERβ mRNA splice variants are regulated by estrogen in the developing rat hippocampus, we gonadectomized rat pups shortly after birth. Immediately following the surgery we treated them with a single injection of 10μg estradiol benzoate in 50μl sesame oil, or oil alone. Following gonadectomy, there is a change in the expression of ERβ mRNA isoforms. On P0, ERβ1 is expressed at the highest relative level, with b1d3 also detected. On postnatal day 7, The pattern of expression of very similar to that at birth with ERb1 expressed at the highest levels. On P15, b1 is still expressed at the highest levels, but at this age following gonadectomy, other ERb splice variants are very low. One exception is the δ4 variant, which is expressed at levels similar to that of the β1 variant.

Following estrogen treatment, the pattern of expression at any given age more closely resembles that of the intact animal such that ERβ1δ4 is expressed at the highest levels early, with δ3 variants increased shortly thereafter. On P10, ERβ2δ3 is expressed at the highest levels, with similar levels of the wildtype form also present.

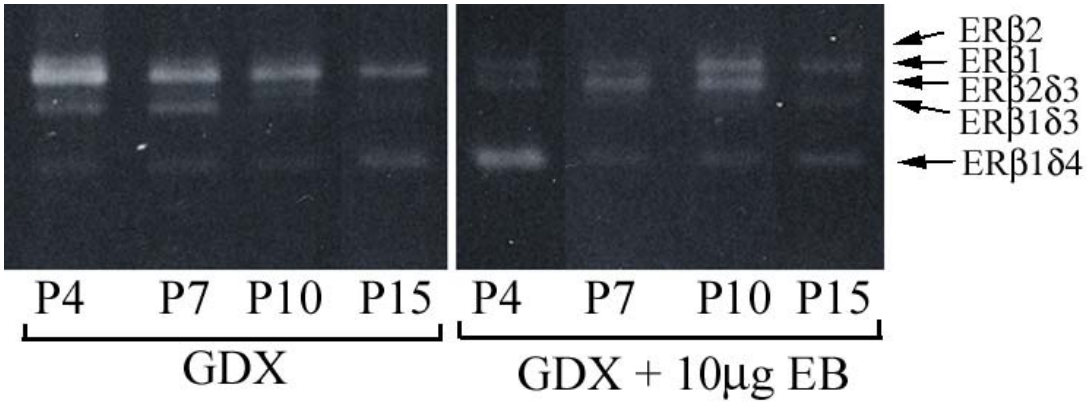



*Figure 20*

Figure 20 legend: Expression of ERβ mRNA splice variants is altered by estrogen in the developing hippocampus. To determine if ERβ mRNA splice variants are regulated by estrogen in the developing hippocampus, we gonadectomized male rat pups shortly after birth. Immediately following the surgery we treated them with a single injection of 10μg estradiol benzoate. Following gonadectomy, there is a change in the expression levels of ERβ mRNA isoforms, where ERβ1 is expressed higher relative to all other isoforms. Following estrogen treatment, the pattern of expression more closely resembles that of the intact animal. ERβ1δ4 is expressed at high levels early, with δ3 variants expressed at high levels shortly thereafter.

gonadectomized animals returns the pattern to one that more closely resembles the intact animal.

Estrogen receptor binding studies demonstrate two binding sites for estrogen in the developing hippocampus, one with high affinity, and one with low affinity

Scatchard analysis with tritiated estradiol ([$^3$H] $E_2$) was performed to determine if estrogen binding during development was consistent with the binding affinities predicted for the mRNA isoforms expressed at same period. Our results presented in figure 21A, B demonstrate that two estrogen binding sites in the neonatal hippocampus exist; one with a low affinity (Kd ~ 5 nM, presumably ERβ2) and one with a much higher affinity (Kd = 0.1nM, presumably ERα, β1). It is unlikely that the change in estrogen binding affinity observed during development is due to alterations in the DNA binding domain present in the δ3 variants, as it has been previously reported that this disruption does not alter the receptor's affinity for estrogen (Petersen et. al., 1998). For these binding studies, we examined [$^3$H] $E_2$ binding in the hippocampus and hypothalamus of P7 and P10 rats. Following analysis, we did not observe a significant difference between these two age groups. Additionally, we did not observe a significant difference between males and females, which were also analyzed separately. We have presented data from P7 males in figure 21A, B.

For comparison, we examined [$^3$H] $E_2$ binding in the hippocampus and hypothalamus of adult females (not shown). There is very little estrogen binding in the

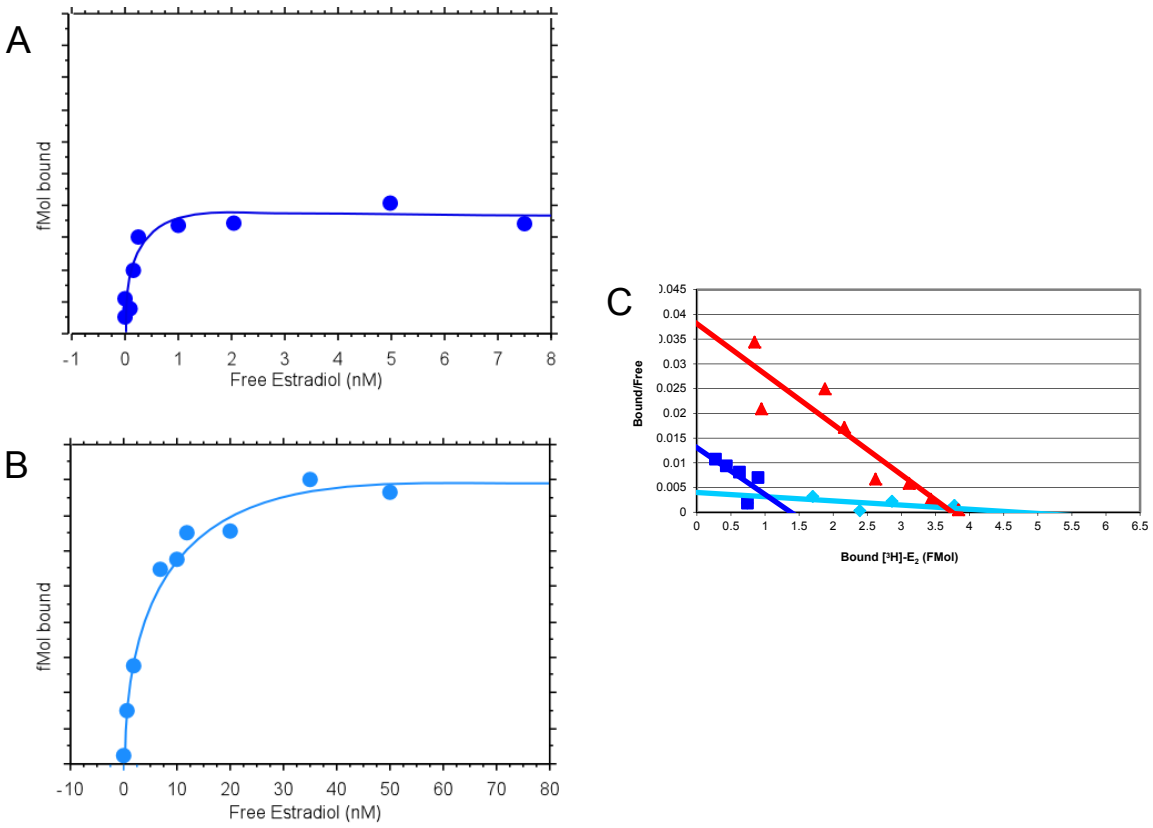

A
fMol bound
-1 0 1 2 3 4 5 6 7 8
Free Estradiol (nM)
B
fMol bound
-10 0 10 20 30 40 50 60 70 80
Free Estradiol (nM)
C
Bound/Free
0.045 0.04 0.035 0.03 0.025 0.02 0.015 0.01 0.005 0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5
Bound [3H]-E2 (FMol)

*Figure 21*

Figure 21 legend: Estradiol binding in the P7 hippocampus. Ligand binding affinity for tritiated estradiol in cytosolic extracts from the hippocampus and hypothalamus of P7 animals was examined. Affinity constants were calculated by nonlinear regression of bound vs. free ligand. Analysis of [$^3$H]$E_2$ binding revealed the presence of two different binding affinities in the P7 hippocampus; one with a low affinity (A; Kd ~ 5 nM, light blue lines) and one with a much higher affinity (B; Kd ~ 0.1nM, dark blue lines). The high affinity binding presumable represents ERα and ERβ1, while the lower affinity binding presumably represents ERβ2. C) Linear transformation of the data by Scatchard analysis is presented for illustrative purposes. Hypothalamic data are presented in the Scatchard analysis for comparison (red line)

adult hippocampus compared to the adult hypothalamus and neonatal hippocampus. This could be due to the fact that we used the entire hippocampus from the adult, and based on recent publications, it appears that estrogen receptors are predominantly located in the ventral hippocampus (Shughrue and Merchenthaler, 2000). It is possible then, that our samples were slightly diluted and thus we didn't observe a very significant level of estrogen binding in the adult hippocampus.

## *Discussion*

In the brain the actions of estrogen are mediated by two different estrogen receptors (ERs); α and β. ERβ mRNA is variably spliced in brain tissue; the protein products of which have been shown to have altered functional properties when expressed *in vitro* (Petersen et al., 1998; Price et. al., 2001). In this study, we examined the expression of ERβ mRNA splice variants in the medial basal hypothalamus, cortex, and hippocampus during early postnatal development. Our results confirm that multiple mRNA variants of ERβ exist in the brain, and that the expression pattern for these variants depends on the brain region and developmental period examined. Interestingly, in the hippocampus there is a developmental change in the levels of ERβ2 mRNA isoforms, where shortly after birth the β2 variants are expressed at very low levels. At P7 there is a dramatic increase in expression, which peaks at P10, then gradually drops to the levels observed in adulthood. Based on conventional PCR methods, we believe the

prominent β2 isoform is the ERβ2δ3 variant also lacking the C-terminal end of the DNA binding domain. Therefore, this variant does not bind DNA or ligand efficiently.

In the hippocampus, gonadectomy at birth results in the expression of ERβ1 at the highest relative levels in each age examined. Following estrogen treatment, the pattern of expression is similar to that of intact animals suggesting that gonadal hormones may regulate the expression of ERβ mRNA splice variants. This has a variety of implications on the developing hippocampus at a period when hormone mediated organizational effects on morphology and physiology are taking place (McEwen, 1983). Interestingly, in both the estrogen and oil treated animals, the levels of all mRNA splice variants gradually decrease over the first two weeks, which could be due to the fact that we only gave a single injection of estrogen. It should also be noted that while the hormone regulation experiments of ERβ splice variants were repeated several times, the results were not always perfectly consistent. Nevertheless, it generally appears that gonadectomy at birth alters the expression pattern of ERβ mRNA variants and estrogen treatment results in an expression pattern similar to intact animals.

In the cortex and hypothalamus, the pattern of expression we observed in the developing animal was similar to that of published reports in the adult (Price et. al., 2000). In these brain regions the wildtype ERβ1 was expressed at the highest relative level at every age examined. In contrast to the hippocampus, we did not observe a significant developmental change in the expression of β2 isoforms, although in the hypothalamus a trend of decreasing β2 expression with increasing age was observed.

However, in the hypothalamus and cortex, we did observe low levels of δ3 and δ4 variants, respectively. Taken together, these data demonstrate that different ERβ mRNA isoforms are present during development in brain tissue and raise interesting questions concerning the physiological significance of ERβ splice variants during development.

In the neonatal hippocampus, the ERβ2δ3 isoform is generally expressed at the highest level. This isoform contains a 54 nucleotide insert in the ligand-binding domain and a deletion of the second zinc finger of the DNA binding domain. Not surprising then, this variant binds ligand with a much lower affinity that the wildtype form (Petersen et. al., 1998; Price et. al., 2001). Our results in the developing hippocampus suggest that an estrogen receptor with altered affinity for estradiol is indeed present. This is observed the first postnatal week using an estrogen binding assay, where two distinct binding affinities are observed; one with a high affinity (Kd ~ 0.1nM) suggestive of the ERα and β1 types, and one with a low affinity (Kd ~ 5nM) suggestive of the β2 isoform. The physiological role of the β2 isoforms are not known. It is possible that estradiol is not the only important ligand for ERβ2. For instance a number of studies have demonstrated that a variety of estrogenic compounds exist which act as agonists with different affinities for the estrogen receptor (Sun et. al., 1999; Petersen et al., 1998). Whether or not this is the case *in vivo* or not remains unclear. Studies to examine the specific expression of ERβ splice variants *in vivo* will require highly specific antibodies that are currently unavailable. Additionally, experiments will be required to determine if some ligands act differentially to regulate the action of estrogen receptors during brain development.

In the past few years, a number of studies have described the presence of alternatively spliced forms of the ERα receptor (Fasco et al., 2000; Pasqualini et al., 2001). However, the majority of these isoforms have only been described in cancer tissues and cell lines (Fugua et. al., 1993; Pfeffer et. al., 1996). Interestingly, the ERβ2δ3 variant that we have shown to be highly expressed in the developing hippocampus, is directly analogous to an ERα exon 3 deletion detected by several investigators (Pfeffer et. al., 1996, Zhang et. al., 1996) and has a similar deletion that truncates the DNA binding domain. The δ3 variants do retain the ability to interact with coactivator proteins such as SRC-1 (Bollig and Miksicek, 2000), suggesting δ3 variants may still may be able to influence DNA transcription through protein protein interactions. This ERα variant has been shown to possess dominant negative activity by inhibiting DNA transcriptional activation by wildtype ERα (Miksicek et. al., 1993). While it is unknown whether the ERβ2δ3 possess dominant negative activity, it is interesting to speculate that this receptor variant could modulate the actions of the wildtype ERβ receptor. Moreover, because ERβ and ERα have been shown to form heterodimers (Cowley et al., 1997), it is even possible that this ERβ2δ3 could influence estrogens actions mediated by the initially described ERα receptor. Considering that ERβ mRNA splice variants are expressed in brain tissue equal to or greater than wildtype ERβ, we believe that these variants should be considered in addition to ERβ1 and ERα when examining the effects of estrogenic agonists and antagonists in nervous tissue.

The biological relevance for the expression of the ER isoforms in normal tissues is not well established. It may be that the expression of ERβ mRNA splice variants in the developing rat hippocampus acts to alter estrogen regulated gene expression, creating a highly complex pattern of estrogen signaling. The expression of ERβ variants with altered ligand or DNA binding properties may functionally act to repress or enhance transcriptional activation by forming dimers with the wildtype receptors. It is also possible that the LBD alterations resulting from mRNA splicing results in receptors that bind ligands with different affinities. These ligands, then, might have either agonistic or antagonistic properties depending on the receptor. At the same time, the ability of splice variants to interact productively with coactivators or other transcription factors gives these variants the potential to stimulate or otherwise modulate gene expression through AP-1 motifs or other DNA binding sites. Further studies will have to be conducted to determine whether the effect of ER mRNA splice variants has a functional relevance to the effects of estrogen on the developing brain.

# CHAPTER 5

## ESTROGEN REGULATES THE DEVELOPMENT OF BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) mRNA AND PROTEIN IN THE RAT HIPPOCAMPUS


### *Abstract*

During development, estrogen has a variety of effects on morphological and electrophysiological properties of hippocampal neurons. Brain-derived neurotrophic factor (BDNF) also plays an important role in the survival and differentiation of neurons during development. We examined the effects of gonadectomy with and without estrogen replacement on the mRNA and protein of BDNF and its receptor, trkB, during early postnatal development of the rat hippocampus. We used immunocytochemistry to demonstrate that estrogen receptor alpha (ERα) and BDNF were localized to the same cells within the developing hippocampus. BDNF and ERα were co-localized in pyramidal cells of the CA3 subregion, and to a lesser extent in CA1. To determine if BDNF mRNA was regulated by estrogen during development, we gonadectomized (GDX) male rat pups at postnatal day (P)0 and examined mRNA and protein levels from P0 to P25 using real-time RT-PCR and Western blot analysis. Following gonadectomy, BDNF mRNA levels are significantly reduced on P7, but after treatment of GDX animals with estradiol benzoate on P0, levels at all ages were similar to intact animals. BDNF mRNA changes following GDX are accompanied by an increase in the levels of BDNF

protein which were reduced by estrogen treatment at P0. We also examined the effect of postnatal estrogen treatment on trkB. There were no significant changes in trkB mRNA or protein in GDX or estrogen replaced animals. These results suggest that a direct interaction may exist between ERα and BDNF to alter hippocampal physiology during development in the rat.

## *Introduction*

It is well established that estrogen is involved in the differentiation and plasticity of hippocampal neurons. For instance, estrogens influence general neurobiological functions such as perceptual-spatial skills and learning and memory (McEwen, 1983; Smith, 1994; Luine, 1997). Estrogens may also act to alter certain specific pathologies such as epileptic seizure threshold (Terasawa and Timiras, 1968; Buterbaugh and Hudson, 1991) and perhaps Alzheimer's disease (Henderson et al., 1996) and Parkinson's disease (Leranth et al., 2000). The sites of action for the effects of estradiol on cognitive performance and pathology have not been established, but one probable site is the hippocampus, a sexually dimorphic, steroid-responsive brain region (Juraska et al., 1989; Roof and Havens, 1992; Woolley and McEwen, 1992).

In the adult rat, it has been demonstrated that hippocampal pyramidal neurons express mRNA for both isoforms (α and β) of the estrogen receptor (Shughrue and Merchenthaler, 2000). Moreover, ovarian steroids act during a perinatal sensitive period to alter the patterns of neuronal cell death and synaptic connectivity (McEwen, 1983).

Additionally, we have demonstrated that ERα is transiently expressed in developing hippocampal pyramidal neurons (Solum and Handa, 2001a). Their biological roles in such processes, however, remain uncertain.

Brain-derived neurotrophic factor (BDNF) is emerging as an important mediator of activity-dependent modifications in synaptic strength (Lohof et al., 1993; Levine et al., 1995) and plays important roles in the survival and growth of neurons (Barde, 1989; Davies, 1994). For instance, BDNF regulates dendritic and axonal growth (Cohen-Cory and Fraser, 1995; McAllister et al., 1995) and the efficacy of synaptic transmission at excitatory synapses on hippocampal neurons (Vicario-Abejon et al., 1998; Sherwood and Lo, 1999).

Interestingly, neurons in the adult rat forebrain of both sexes co-express estrogen and neurotrophin receptors and are the sites of estrogen and neurotrophin synthesis (Toran-Allerand et al., 1992; Miranda et al., 1994). Consistent with this, the relative levels of BDNF mRNA within specific regions of the hippocampus fluctuate significantly over the course of the estrous cycle (Gibbs, 1998) and increased levels of BDNF mRNA following long-term estrogen treatment have been reported in this brain region (Singh et al., 1995). It has also been shown that estrogen and neurotrophin receptor co-expression leads to convergence of their signaling pathways (Toran-Allerand et al., 1999). However, whether estrogen influences BDNF or trkB expression in the developing hippocampus *in vivo* and whether the developmental actions of estrogen on neurons are mediated directly or indirectly via interactions with growth factors and their signaling pathways is unclear.

Given the increasing evidence for effects of BDNF on neuronal connectivity and activity dependent synaptic plasticity in the adult brain, we hypothesized that estrogen could similarly effect BDNF expression during development. This may subsequently contribute to estrogen's organizational effects on brain structure and function. In this study, we examined the effect of gonadectomy and estrogen treatment on the developmental expression of mRNAs and protein for BDNF and its receptor, trkB, using immunocytochemistry, real-time quantitative RT-PCR and western blot analysis.

## *Materials and Methods*

Animals. Neonatal male and female rats of various ages from timed-pregnant Sprague-Dawley females and adult male and female adult rats were obtained from Charles River Laboratories (Portage, MI). Animals were housed under a 12/12 h light dark cycle (lights on at 07.00 h) with food and water available *ad libitum*. Upon parturition, litters were sexed and thinned to 8 pups (4 males and 4 females). Shortly after birth (4-6 hours), some pups were gonadectomized under hypothermia anesthesia. Of these, half received a single subcutaneous injection of 10μg estradiol benzoate in 50μl sesame oil, while the other half received oil alone. This protocol has been shown to result in a decrease in plasma testosterone levels, an increase in estrogen receptor levels and the abolishment of male sexual behavior (Ulibarri et al., 1990; Kuhnemann et al., 1995; McCormick et al., 1998; Atanassova et al., 1999), presumably by overcoming the sequestering ability of circulating alpha-fetoprotein. Animals were subsequently sacrificed at postnatal (P) days

4, 7, 10, 15, 20 and 25 (day of birth = P0). Adult males and females were sacrificed at 3-4 months of age. An additional group of intact animals were sacrificed on P0. All animal protocols were previously approved by the Animal Care and Use Committee at Colorado State University.

Hippocampal cultures. Neuronal cultures were prepared from rat embryonic hippocampus at embryonic day (E)18. Embryos were recovered following maternal c-section under halothane anesthetic and the hippocampi were dissected from the brain and minced. Individual cells were isolated by trituration in Hanks' Balanced Salt Solution (HBSS; Gibco BRL, Rockville, MD) without $Ca^{++}$ and $Mg^{++}$. After allowing nondispersed tissues to settle for 3 min, the supernate was transferred to a sterile 15mL tube and centrifuged for 1 min at 200 x g. The pellet was gently resuspended in charcoal stripped media without phenol red and an aliquot added to trypan blue stain for a haemocytometer count. Cells were then plated on poly-D-lysine (0.05mg/ml; Sigma, St. Louis, MO) coated coverslips at a density of 50,000 cell/$cm^2$ and incubated at 37°C in 5% $CO_2$ atmosphere. After four days in culture, one half of the media was replaced and the cultures were treated with estradiol benzoate (0.01, 0.1, 1.0, 10 and 100nM) or vehicle for 24 hours before processing.

Antibodies. Anti-ERα antibodies, raised against the 14 most carboxyl terminal amino acids, were purchased from Upstate Biotech (C1355; Lake Placid, NY); anti-ERβ antibodies were from Zymed Laboratories and were raised against amino acids 468-485 of the ERβ protein (Z8P; San Francisco, CA); gamma amino butyric acid (GABA;

A2052) antiserum was obtained from Sigma; BDNF (sc-546) and trkB (sc-12) antiserum were from Santa Cruz Biotech (Santa Cruz, CA) and map to the amino and carboxyl terminal regions, respectively. To ensure immunoreactive specificity, competition experiments were performed using the immunoreactive peptides for BDNF (sc-546P) and trkB (sc-12P).

Double-label immunocytochemistry. Neonatal rat pups were anesthetized with Halothane and sacrificed by perfusion with 10-30mL of 0.1M ice-cold phosphate buffered saline (PBS) followed by 10-30mL of freshly prepared ice-cold 4% paraformaldehyde in 0.1M PBS. Adult animals were anesthetized and sacrificed similarly, but were perfused with approximately 200mL of 0.1M PBS followed by 200mL of freshly prepared 4% paraformaldehyde in 0.1M PBS. Brains were removed and placed into 30% sucrose in 0.1M PBS at 4°C until permeated. Next, the brains were sectioned 30μm thick on a cryostat and placed into 0.1M PBS containing 0.1% Sodium Azide. The tissue was processed for immunocytochemistry (ICC) as described previously (Kerr et al., 1995). Briefly, tissue from three P10 males was incubated with 0.3% $H_2O_2$ in 0.1M PBS to quench any nonspecific reaction with endogenous peroxidases. Subsequently, non-specific antibody binding was blocked by incubation with 4% normal goat serum (NGS) in 0.1 M PBS. Tissue was then incubated for 48 hours at 4ºC with ERα (1:10,000) or ERβ (1:1,500) antiserum in the presence of 2% NGS and 0.1% Triton X-100 (TX). The tissue was then washed and incubated for 2 hours with biotinylated goat anti-rabbit IgG (1:500; Vector Labs, Burlingame, CA) followed by standard washes and incubation with

an avidin-biotin-horseradish peroxidase complex (1:500; Vector Labs) for 1 hour. Standard washes were done three times at room temperature in 0.1M PBS with 0.1% TX. Finally, staining was visualized with a tris-buffered saline solution containing nickel intensified diaminobenzadine (DAB, 0.5mg/ml; Sigma) and hydrogen peroxide (0.01%) for 6-10 minutes. Nickel intensified DAB was prepared by adding 0.3mg/mL of nickel ammonium sulfate to the DAB solution resulting in a dark blue stain.

Next, the tissue was washed and processed for BDNF immunoreactivity using BDNF antiserum (1:500). Essentially, the tissue was processed as above, except that after incubating with 0.3% $H_2O_2$ the tissue was blocked for 2 hours in 6% NGS. Tissue was then washed (3 x 15 min. in 0.1M PBS-TX) before the secondary antibody incubation as described above. Following a final washing step, this reaction was developed with normal DAB (0.5mg/mL + 0.01% $H_2O_2$) to produce a brown reaction product. After developing, the tissue was rinsed and mounted on glass slides. The mounted sections were air-dried overnight at room temperature and the slides were processed through a series of increasing alcohols, cleared with xylene, and coverslipped with Permount (Fisher Scientific). Double-labeled cells were visualized by light microscopy as cells containing a dark blue nucleus (ERα) and a brown cytoplasm (BDNF).

Fluorescent double-label immunocytochemistry on dissociated hippocampal cells was essentially similar to that described above with some exceptions. After five days *in vitro*, culture media was removed and the cells were fixed with 10% buffered formalin for 10 minutes after which the cells were washed three times in PBS-TX. Following

blocking and primary antibody incubations similar to that described above, the cells were washed and incubated for two hours with Alexa Fluor 594 goat anti-rabbit IgG conjugate (1:2000; Molecular Probes, Eugene, OR). Cells were then washed and incubated with unlabeled goat anti-rabbit IgG (6% in PBS-TX) for an hour to block remaining binding sites on ER antibodies. The cells were then processed for the second primary antibody as described above with similar blocking, primary and secondary antibodies incubations. Following another washing step, cells were incubated for one hour with Alexa Fluor 488 streptavidin conjugate (1:1,500; Molecular Probes). After a final washing step, the coverslips were affixed to Superfrost Plus slides with Vectashield mounting medium (Vector Laboratories). Cells were analyzed with a Zeiss Axioplan 2 imaging microscope and images were captured with a Zeiss AxioCam digital camera.

Total RNA isolation and reverse transcription. Neonatal rat pups were decapitated, and the brains quickly removed and the medial basal hypothalamus and hippocampal regions CA1 and CA3 were dissected. Total RNA was isolated similar to the protocol of (Chomczynski and Sacchi, 1987). Briefly, brain tissue was homogenized in 250µl of GIT buffer (4M guanidinium isothiocyanate/25mM NaCitrate, pH 7.0/0.5%sarcosyl/0.1M β-mercaptoethanol) on ice. Subsequently, 25µl of 2M NaOAc (pH 4.0), 250µl of buffer-saturated phenol (pH 4.3) and 50µl of chloroform/isoamyl alcohol (49:1) was added and the mixture vortexed. The samples were then centrifuged at 14,000 x g for 20 minutes at 4ºC. The aqueous phase was recovered and the RNA was ethanol precipitated. The resulting RNA was washed with ice-cold 70% ethanol, and reconstituted in 50µl RNAse

free water. The RNA content was measured with a spectrophotometer and only those samples with a 260/280 ratio above 1.6 were used.

2 μg of total RNA was reverse transcribed with Superscript II reverse transcriptase (RT; Gibco/BRL, Rockville, MD) using oligo-dT primers, deoxyribonucleotides (dNTPs; 100 mM each), 1st strand buffer (100 mM Tris-Cl/900 mM KCl/1 mM $MgCl_2$) and 2.5 mM dithiothreitol (DTT). The reaction was carried out at room temperature for 10 minutes followed by 50 minutes at 42ºC. The RT was then denatured at 95ºC for 10 minutes and stored at –80ºC until used.

Real-time quantitative polymerase chain reaction (PCR). Real-time quantitative PCR was performed using the LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN). In this system, PCR occurs in borosilicate glass capillaries which have a high surface-to-volume ratio to ensure rapid equilibration between the air and the reaction components. A highly specific double-stranded (ds) DNA binding dye, SYBR Green I (Molecular Probes, Eugene, OR), which only fluoresces when bound to dsDNA, is used to determine the concentration of amplified products. SYBR Green I binds to the minor groove of dsDNA and fluorescence is greatly enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending the amount of dsDNA that is present. The 530 nm fluorescence is recorded at the end of the elongation phase, and increasing amounts of PCR product are monitored from cycle to cycle. By comparing the amount of unknown cDNA to a curve of amounts of a given cDNA amplified concurrently, real-time PCR eliminates the need for competitive in-tube standards with identical primer sets as targets (Morrison et al., 1998).

To prevent nonspecific amplification we used hot-start PCR with dNTPs, specific primers, PCR buffer [100 mM Tris-Cl, 1.5 mM $MgCl_2$, 0.5 units Taq polymerase, 0.5 units Taq antibody (Gibco/BRL) and 2 μl of 10X stock SYBR Green I]. Specific primers were as follows: BDNF (Genbank accession #M61178) forward primer position 245, reverse primer position, 711; trkB (Genbank accession #M55291) forward primer 2091, reverse primer 2406. Primers were developed using OLIGO software (v. 6.51; Molecular Biology Insights, Cascade, CO). All samples were amplified at 40 cycles which is approximately five to ten cycles beyond the beginning of the linear phase of amplification. Specifically, an initial melting step was done at 95ºC for 2 minutes followed for 40 cycles of 95ºC melting step for 1s, an annealing step (60ºC for trkB and 64ºC for BDNF) for 5s, and a 72ºC elongation step (13s for trkB and18s for BDNF). In all experiments, samples containing no template were included to serve as negative controls. To ensure that the standard and unknown samples amplified equivalently additional control experiments were conducted in which a known amount of BDNF or trkB cDNA (from the standard curve) was added to the unknown samples. These results were then compared to the sum of those obtained from the known and unknown samples amplified independently and were not significantly different ($p = 0.797$).

Construction of the BDNF and TrkB standard curve. To determine the absolute concentration of the target transcript, conventional PCR for BDNF and TrkB was used to generate a cDNA. The amplified cDNA was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) according to the manufacture's directions. The purified PCR

products were serially diluted at a range of 30ng-30fg and this curve was run in duplicate alongside the unknown samples.

mRNA quantitative analysis. Following real-time PCR, the absolute concentration of mRNA in each sample was determined by analysis with LightCycler Data Analysis (LCDA) software. This software plots a standard curve of the crossing line intercepts of the standards vs. the known concentrations of these standards. The crossing line intercept is parallel to the x-axis on a graph of fluorescence intensity vs. cycle number and occurs at the point where template amplification enters the logarithmic phase of the curve. Samples with a higher concentration of starting material enter the logarithmic phase earlier than samples with a lower concentration of starting material and therefore have a smaller crossing point value. The crossing line intercept of an unknown sample is subsequently compared with the standard curve to generate a quantitative amount of starting material. In each case, the point at which the crossing line intercepts the log-linear region of each curve is used to generate the concentration of that sample.

Western Blot Analysis. Animals were sacrificed at ages described above and the brains were quickly removed. The hippocampi were dissected and individually homogenized in 200µl of 50mM Tris buffer (pH 7.2, 4ºC) containing 1mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM leupeptin, 1 µg/ml antipain, and 1 µg/ml aprotinin. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Homogenates were mixed 1:1 with sample buffer containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.05% bromphenol blue and boiled for 5

minutes. For each age, 20μg of protein were loaded into each well. The samples were separated on 8% (trkB) or 12% (BDNF) SDS polyacrylamide gels along with biotinylated molecular weight standards (Bio-Rad, Hercules, CA). Following electrophoresis, the proteins were electrically transferred to nitrocellulose in 49.6mM Tris, 384 mM glycine, 0.01% SDS at 30V overnight followed by 80V for 1 hour. The gels then were stained with Coomassie blue to confirm equal loading per lane. After transfer, blots were incubated in tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% non-fat milk, 2% bovine serum albumin, and 0.1% sodium azide. Subsequently, blots were incubated with either BDNF or trkB (1:500) antiserum for 48 hrs. at 4ºC in TBST containing 2% non-fat milk and 0.1% sodium azide. Blots were also processed without primary antibodies or antibodies that had been preabsorbed with the immunoreactive peptide to serve as controls. Following primary antibody incubation, the blots were washed (three times, 15 minutes each in TBST at 25ºC) and incubated with horseradish peroxidase conjugated goat anti-rabbit antibodies (1μg/mL, Vector Labs, Burlingame, CA). Immunoreactive bands were visualized with an enhanced chemiluminescence system (ECL, Amersham, Arlington Heights, IL) according to manufacturer's directions. To ensure that each lane was loaded with an equivalent amount of protein, the blots were stripped with 0.2M NaOH and re-probed with anti-actin serum (1:10,000; Chemicon, Temecula, CA) as described above. Following immunoblotting, digitized images of immunoreactive bands for target (BDNF, trkB) and control (actin) products were imported into NIH Image software (v. 1.62) and the average OD of each band was measured (based on a grayscale of 0-256 arbitrary units, 0 being white and 256 being

black). Additional background measurements were taken from each film and subtracted from these values. A ratio of BDNF, trkB/actin was then determined and these values were compared across development for statistical significance.

Statistical analyses. All analyses were performed using multi-way analysis of variance (ANOVA, StatView, SAS Institute Inc.). Significant values were subsequently verified with the Tukey-Kramer post hoc analysis.

## *Results*

### Estrogen receptor alpha, but not beta, co-localizes with BDNF in hippocampal pyramidal cells in vivo and in vitro

In our initial studies we sought to determine if estrogen receptors alpha and/or beta were co-localized with BDNF in neurons of the developing rat hippocampus using double label immunocytochemistry. We demonstrated that ERα and BDNF were highly co-localized in pyramidal cells of the CA1 and CA3 hippocampal subregions of the intact male and female rat on postnatal day 10 (Fig. 22). We chose this age based on our previous experiments showing that while little or no ERα positive cells are observed on postnatal day 0, a transient increase in ERα occurs from postnatal day 4 to 10 (Solum and Handa, 2001a). Our results, presented here, demonstrate that ERα and BDNF were co-localized in stratum pyramidale of the CA3 subregion and to a lesser extent in the CA1 subregion. Double-labeled neurons are visualized as cells with a dark blue nucleus and a

brown cytoplasm. Additionally, a few scattered ERα immunoreactive neurons were also observed in stratum radiatum and oriens. However, these cells did not express BDNF as well, and are likely non-pyramidal interneurons.

Next we examined whether ERβ was also co-localized with BDNF in the developing rat hippocampus. While we observed a few neurons in which ERβ and BDNF were co-localized in the neonatal hippocampus, the majority of BNDF immunoreactive cells were ERβ negative (Fig 22). In all of the samples we examined, the intensity level of ERβ immunoreactivity was much less than that for ERα. Additionally, in each of these experiments we did not observe an obvious sex difference in the number or intensity of immunoreactivity for ER (α and β) or BDNF.

It is difficult, however, to determine the extent to which cells are double-labeled due to the fact that BDNF expressing pyramidal cells are densely packed in stratum pyramidale of the hippocampus. Because of this, we examined whether BDNF and ERs are co-localized in primary hippocampal cells grown at moderate density *in vitro* for

Figure 22 Legend: Double-label immunocytochemistry of estrogen receptor (α and β) and BDNF in the neonatal hippocampus. Double-label immunocytochemistry in the P10 male hippocampus was used to co-localize ERα and BDNF to pyramidal neurons in the CA1 (A) and CA3 (B) hippocampal subregions. ERα immunoreactivity is visualized as a blue nuclear stain, while BDNF immunoreactivity is visualized as a brown cytoplasmic stain. The majority of immunopositive cells were $ER\alpha^{+}/BDNF^{+}$ (arrows and inset) although some $ER\alpha^{-}/BDNF^{+}$ cells (arrowheads) cells were also observed. ERβ and BDNF were not appreciably co-localized in either the CA1 (C) or CA3 (D) hippocampal subregions of the intact P10 male rat. A few $ER\beta^{+}/BDNF^{+}$ (arrows) cells were observed but the majority were $ER\beta^{-}/BDNF^{+}$ (arrowheads). Scale bar = 50μm

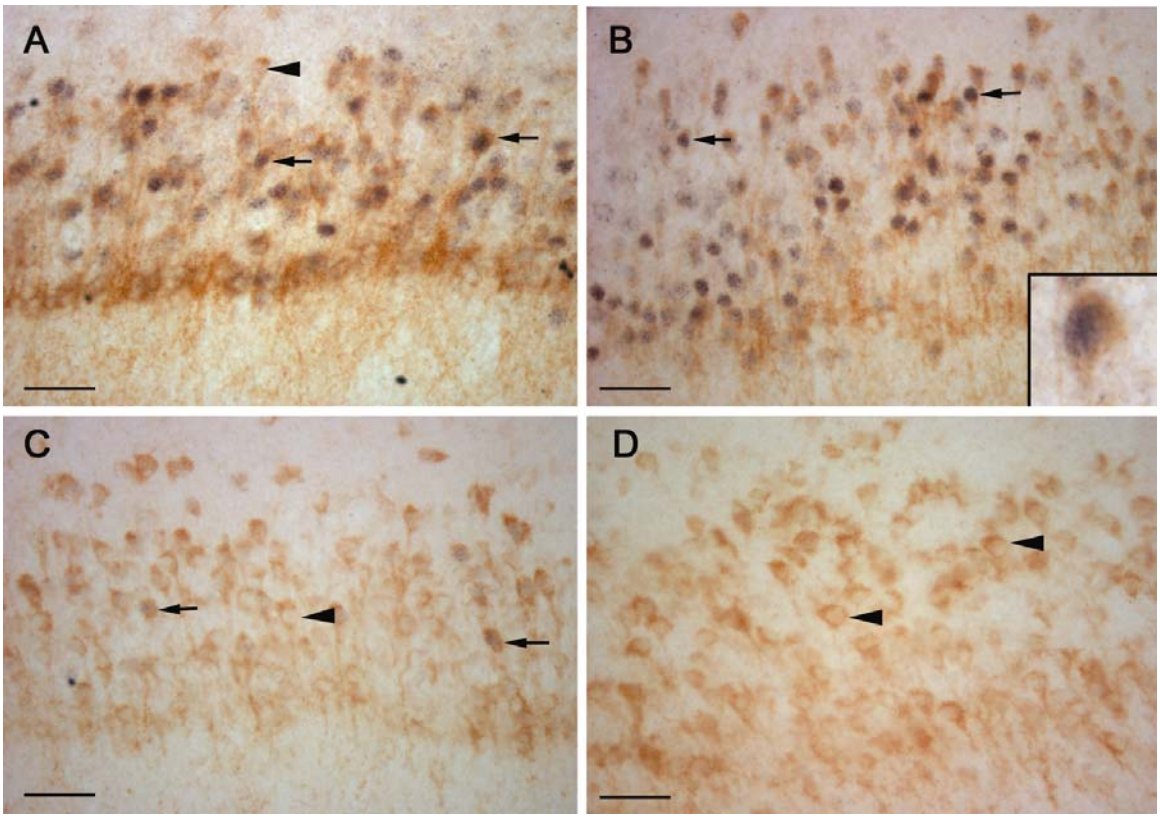



*Figure 22*

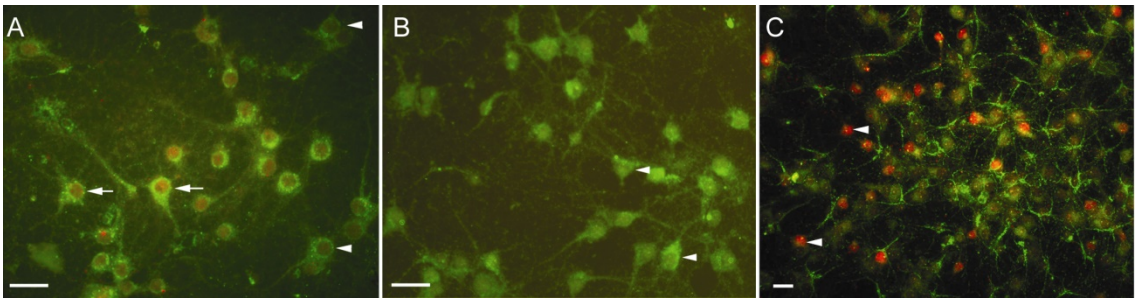



*Figure 23*

Figure 23 Legend: Co-localization of ERs with BDNF and GABA in primary cultures of hippocampal cells. Double-label fluorescent immunocytochemistry in embryonic hippocampal cell cultures grown *in vitro* for five days was used to co-localize ERα, but not ERβ, and BDNF. Each figure is an overlay of ERα or β and BDNF or GABA immunocytochemistry. ER immunoreactivity is visualized as red immunofluorescence, while BDNF or GABA immunoreactivity is green. (A) The majority of immunopositive cells were $ERα^{+}/BDNF^{+}$ (arrows), although some $BDNF^{+}$ cells were observed which expressed little or no ERα-ir (arrowheads). (B) Essentially no ERβ immunoreactivity was observed to be co-expressed with BDNF and the nuclei of these cells are essentially unstained (arrowheads). (C) No cells were observed that express ERα and GABA, although ERα positive cells (arrowheads) and GABAergic processes (arrows) clearly visible. Scale bar = 25μm

five days. Additionally, we double labeled primary hippocampal cultures with ERα and GABA to confirm our previous findings *in vivo* showing ERα is primarily located in

pyramidal cells, not interneurons. These experiments confirmed our findings *in vivo* and demonstrated that ERα, but not ERβ, was co-localized with BDNF in hippocampal pyramidal cells grown *in vitro* (Fig. 23). In this experiment, the majority of immunopositive cells were $ER\alpha^{+}/BDNF^{+}$, although some $ER\alpha^{-}/BDNF^{+}$ cells were also present. We did not observe any $ER\alpha^{+}/BDNF^{-}$, $ER\beta^{+}/BDNF^{+}$ or $ER\alpha^{+}/GABA^{+}$ cells.

BDNF mRNA levels are decreased in the hippocampus following neonatal gonadectomy and this effect can be reversed by a single injection of estrogen

Using quantitative real-time RT-PCR we examined the expression of BDNF messenger RNA in the CA1 and CA3 hippocampal subregions of the intact male rat during development and compared these values to those from males that had been castrated on postnatal day 0 and immediately given a single injection of oil vehicle or estradiol benzoate (Fig. 24). Because injected estrogen levels have a longer half-life in newborn rats than in older animals (MacLusky et al., 1979), we used this experimental protocol to mimic the increase in brain estrogen levels observed early in postnatal life. This allowed us to examine the direct organizational effects of estrogen on BDNF, rather than indirect effects resulting from the aromatization of testosterone. Our results demonstrate that the level of BDNF mRNA expression increases in the intact animal from P4 to P7 in the CA1 region, and P4 to P10 in the CA3 region ($p < 0.001$), and then

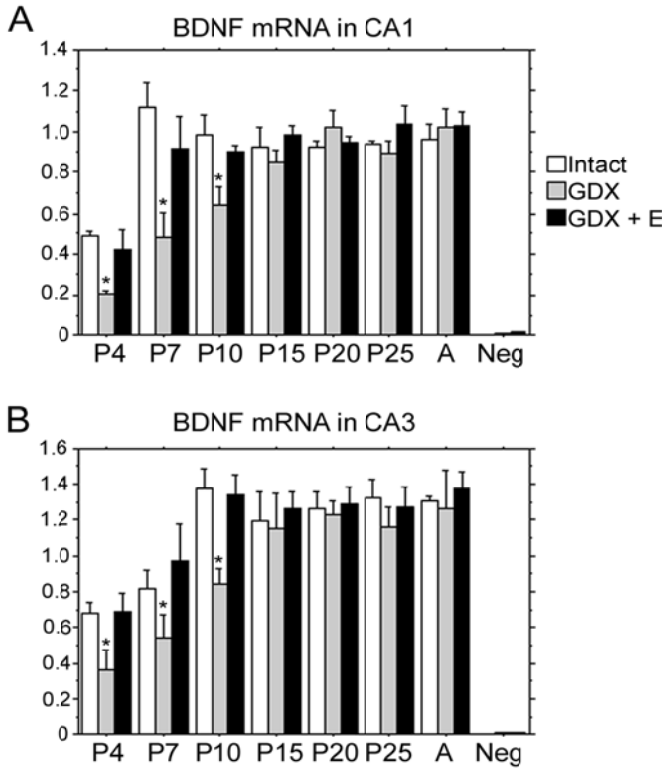



*Figure 24*

Figure 24 Legend: BDNF mRNA levels in the CA1 and CA3 hippocampal subregions of the developing male rat following castration and estrogen replacement. Quantitative real-time RT-PCR was used to examine the expression of BDNF messenger RNA in the CA1 and CA3 hippocampal subregions of the male rat during development. Animals were gonadectomized on P0, and given a single injection of estradiol. All values are reported in picograms of cDNA. Each bar represents the mean of three independent experiments ± S.E.M. (*) Significantly less than other groups at that age ($p < 0.005$). A = adult, Neg = negative control

is maintained through adulthood. However, when males are castrated at birth, developing levels are significantly attenuated in these hippocampal subregions. Beginning on P4, and continuing through P10, the levels of BDNF mRNA are significantly less in the hippocampus of castrated males compared to intact controls (CA1, $p = 0.002$; CA3, $p = 0.003$). By P15, the levels of BDNF mRNA from castrated animals are no longer significantly different from intact males in either the CA1 or CA3 subregion, and this remains the case into adulthood. When neonatally castrated males are given a single injection of estradiol benzoate, the levels of BDNF mRNA expression are significantly higher in the CA1 and CA3 hippocampal subregions from castrated males given vehicle alone ($p < 0.001$). Moreover, the levels of BDNF mRNA expression are not different from intact animals (CA1, $p = 0.609$; CA3, $p = 0.536$). Even so, in each of these treatment groups, a developmental trend similar to intact animals was observed where BDNF mRNA levels increased during the first two postnatal weeks at which point they were not different from adults. Also important to note is that the time course for estrogen's effects on BNDF mRNA expression are very similar to the transient increase in estrogen receptors in the hippocampus that we have previously reported. These data demonstrate that estrogen significantly increases BDNF mRNA levels in the hippocampus during development and this effect is likely mediated through estrogen receptor alpha.

Estrogen effects BDNF mRNA expression *in vitro* in a dose dependent manner

To determine at what concentration estrogen effects on BDNF mRNA expression are observed we treated primary hippocampal cells with various doses of estrogen ranging from 0.01nM to 100nM (Fig. 25). Following estrogen treatment we examined BDNF mRNA expression with quantitative real-time PCR. Our results demonstrate that estrogen regulates BDNF mRNA expression in a dose dependent manner. Very low doses (0.01nM) of estrogen had no effect, while increasing doses resulted in an upregulation of mRNA levels. Treatment with 10nM estrogen resulted in a significant increase in BDNF mRNA over control levels, and this was only slightly elevated with higher concentrations. These results confirmed our findings *in vivo* and demonstrate that estrogen significantly upregulates BDNF mRNA levels in hippocampal cultures. Additionally, these experiments provide evidence to support the utilization of primary hippocampal cultures as a model for studying effects of estrogen on BDNF expression.

BDNF mRNA levels in the hypothalamus are unaffected by gonadectomy or estrogen replacement

Previous studies have shown that the effect of estrogen on BDNF mRNA expression in the adult varies depending on the brain region examined (Jezierski and Sohrabji, 2000). To determine whether estrogen affects BDNF mRNA levels in a brain region specific manner during development, we examined the medial basal

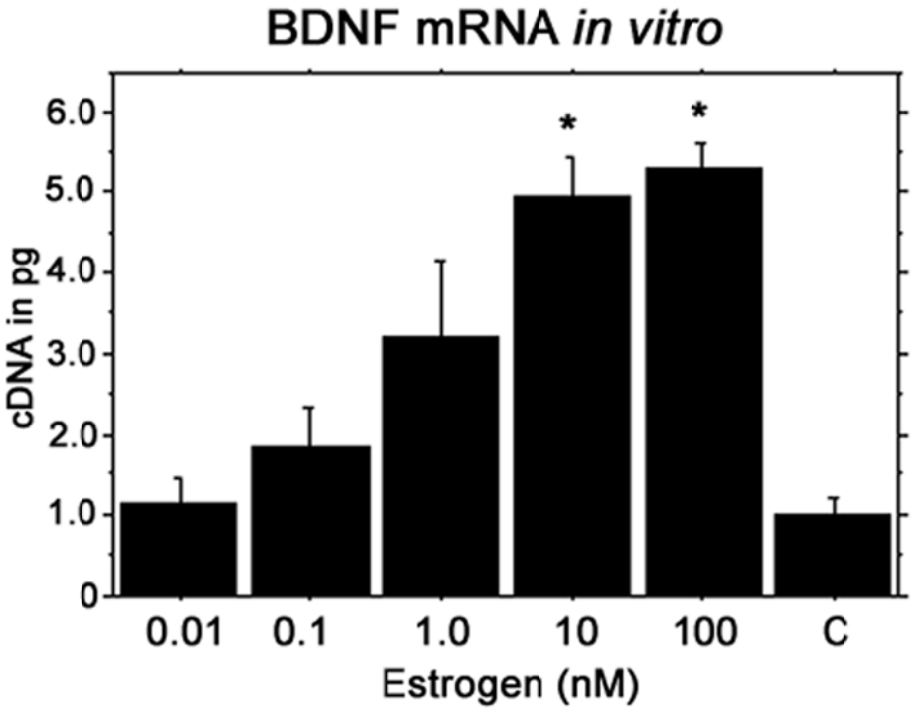



*Figure 25*

Figure 25 Legend: Effects of estrogen on BDNF mRNA expression in primary hippocampal cultures. Primary hippocampal cultures were treated with increasing doses of estradiol benzoate for 24hours, after which BDNF mRNA levels were examined with real-time PCR. All values are reported in picograms of cDNA. Each bar represents the mean of four independent measurements ± S.E.M. (*) Significantly greater than other controls ($p < 0.002$). C = control

hypothalamus in addition to the hippocampus. The existence and distribution of BDNF mRNA within the adult rat hypothalamus has been well established (Marmigere et al., 1998). Using quantitative real-time PCR we examined the expression of BDNF mRNA in the developing hypothalamus of intact male rats and neonatal male rats castrated at birth and given a single dose of either 10μg estradiol benzoate or vehicle. Our results demonstrate that BDNF mRNA levels in the hypothalamus of developing male rats were unaffected either by neonatal castration or castration plus estrogen replacement (Fig. 26). Interestingly, we did not observe a significant change across development and the level of BDNF mRNA expression was generally lower in the hypothalamus when compared to the hippocampus. These findings are similar those observed in the avian hypothalamus, where neither acute nor chronic treatment of 17β-estradiol had an effect on BDNF mRNA levels (Viant et al., 2000).

Neonatal gonadectomy increases BDNF protein levels in the developing hippocampus and this effect can be reversed by a single injection of estrogen

After observing an effect of estrogen on BDNF mRNA expression in the developing hippocampus, we next examined whether estrogen had an effect on BDNF protein levels in this brain region as well. We used Western blot analysis to compare the effect of estrogen on BDNF expression in the developing hippocampus of normal males, males castrated at birth and castrated animals given an injection of estradiol benzoate at the time of castration. These data are presented in Fig. 27. In the intact

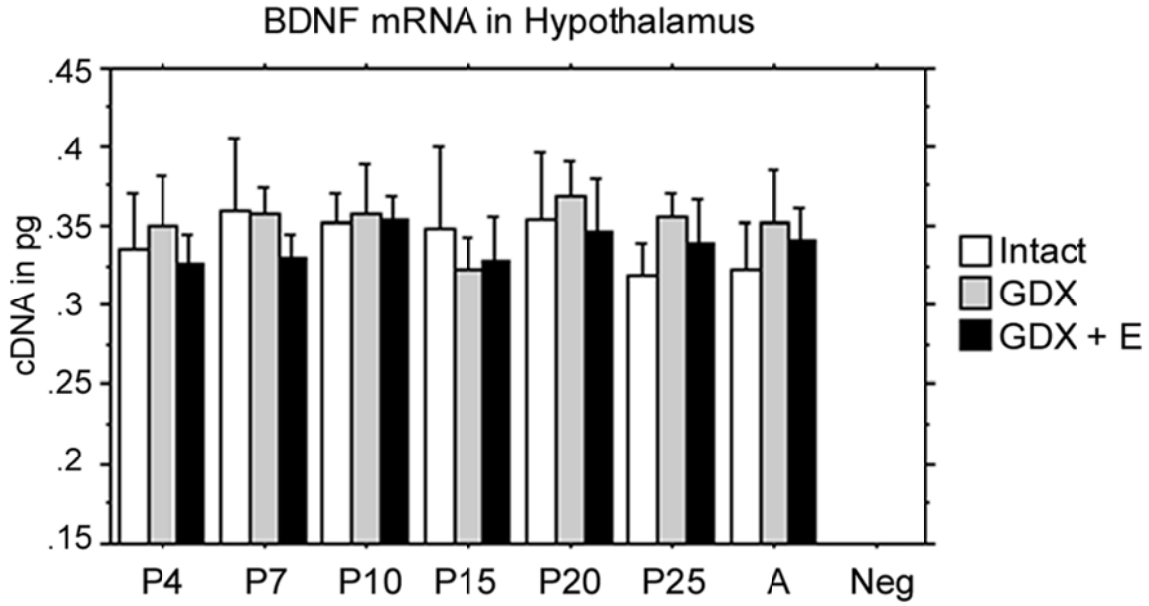



*Figure 26*

Figure 26 Legend: BDNF mRNA levels in the medial basal hypothalamus of the developing male rat following castration and estrogen replacement. Real-time RT-PCR was used to quantitate the expression of BDNF messenger RNA in the medial basal hypothalamus of the male rat during development. Animals were gonadectomized on P0, and given a single injection of oil vehicle or estradiol benzoate. All values are reported in picograms of cDNA. Each bar represents the mean of three independent experiments ± S.E.M. No significant differences were observed across development or between treatment groups ($p > 0.99$). A = adult, Neg = negative control

Figure 27 Legend: Estrogen regulation of BDNF levels in the developing hippocampus. BDNF levels were measured using western blot analysis. (A) A single band of approximately 14kDa was detected in hippocampal protein samples from intact, gonadectomized (GDX) and estrogen treated males during various stages of postnatal development. Males were GDX on postnatal day 0 and immediately treated with a single injection of oil vehicle or estradiol benzoate. (B) Following Western blot analysis of BDNF and actin in the developing hippocampus of intact and hormone manipulated males, mean optical densities of immunoreactive bands were determined. Background levels were subtracted from these measurements and a ratio of BDNF/actin was determined to give a mean net density for each age. Each bar represents the mean ± S.E.M. of three independent experiments. (*) Significantly greater than other treatment groups at that age ($p < 0.001$).

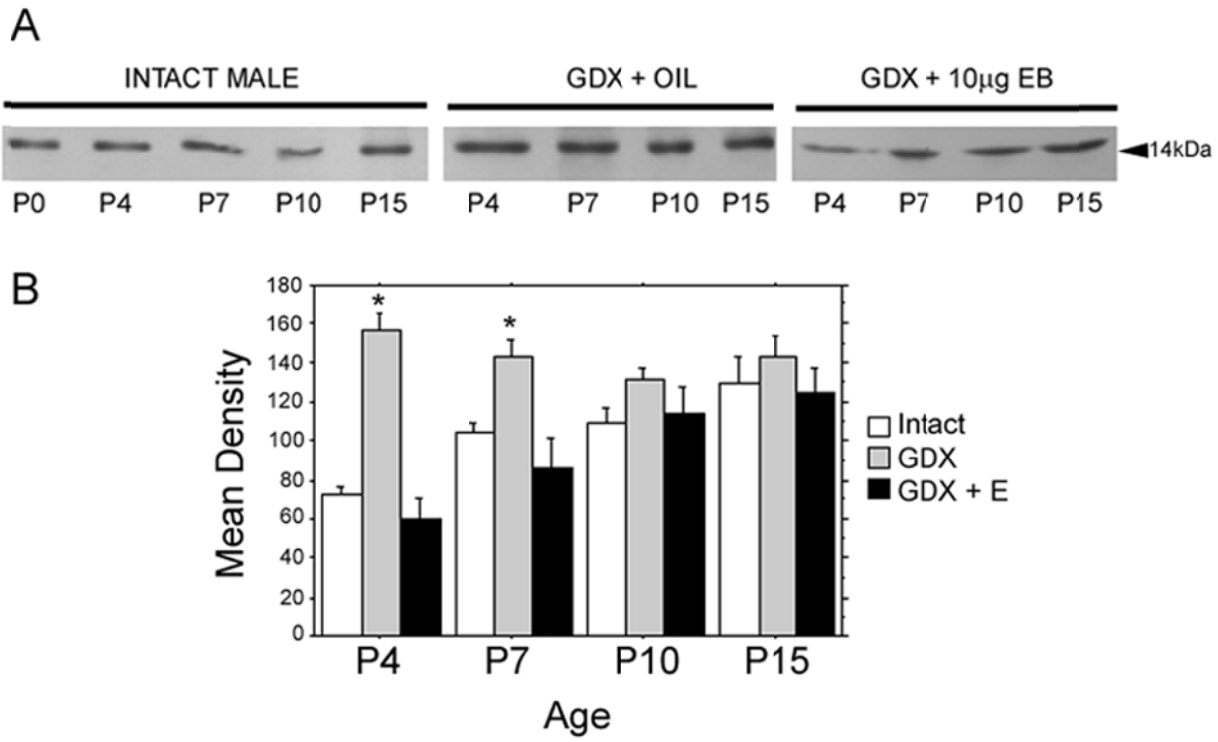

A
INTACT MALE
GDX + OIL
GDX + 10µg EB
14kDa
P0
P4
P7
P10
P15
P4
P7
P10
P15
P4
P7
P10
P15
B
Mean Density
180
160
140
120
100
80
60
40
20
0
*
*
P4
P7
P10
P15
Age
Intact
GDX
GDX + E

*Figure 27*

animal we observed a developmental trend in BDNF protein levels similar to that of BDNF mRNA where levels increased from P0 to P10 ($p < 0.003$). However, contrary to our expectations, neonatal castration did not decrease BDNF protein levels as it had with mRNA levels. Quite the opposite, from postnatal day 4 to 7, BDNF levels in the hippocampus were significantly increased following castration at birth ($p < 0.001$). Moreover, when castrated animals were given a single injection of 10μg estradiol benzoate, BDNF protein levels were no longer significantly different from intact males. To ensure that the immunoreactive bands that we observed were specific for BDNF, we conducted two control experiments including elimination of the primary antibody and preadsorption of the antiserum with the immunoreactive peptide (100ng/ml; sc-546P, Santa Cruz Biotech). Both of these control experiments completely eliminated the immunoreactive band (data not shown).

Estrogen does not effect trkB mRNA or protein expression in the developing hippocampus

To determine whether estrogen alters neuronal sensitivity to BDNF by influencing BDNF receptor concentration, we examined the mRNA and protein expression for trkB, the high affinity BDNF receptor. Using quantitative PCR and Western blot analysis we did not observe any differences in mRNA (Fig. 28) or protein (Fig. 29) expression in the hippocampus across development when comparing normal males with castrated males treated with estradiol benzoate or vehicle. We analyzed trkB mRNA in both the CA1 and CA3 hippocampal subregions from postnatal days 4 to

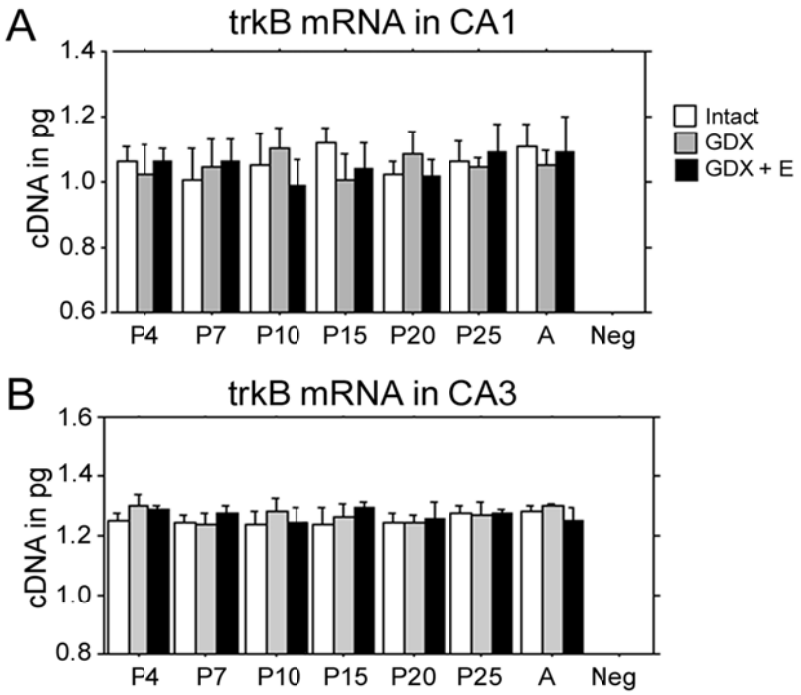



*Figure 28*

Figure 28 Legend: trkB mRNA levels in the CA1 and CA3 hippocampal subregions of the developing male rat following castration and estrogen replacement. Quantitative real-time PCR was used to examine the expression of trkB messenger RNA in the CA1 and CA3 hippocampal subregions of the male rat during development. Animals were gonadectomized on postnatal day 0 and given a single injection of oil vehicle or estradiol benzoate. All values are reported in picograms of cDNA. Each bar represents the mean ± S.E.M.of three independent experiments. No significant changes occurred following castration or estrogen replacement at any age examined ($p > 0.810$).

Similar to earlier reports using a different technique (Fryer et al., 1996), we detected very little change in expression during the first two postnatal weeks, where the levels of mRNA for full length trkB are no different from adult levels (Fig. 7). Additionally, we did not observe a significant difference between any treatment group at each timepoint ($p = 0.680$).

Next, we examined trkB protein expression from postnatal days 4 to 15 in similar treatment groups (Fig. 8). After immunoblotting with anti-trkB serum, we observed an immunoreactive doublet at approximately 145kDa. The larger band of this doublet represents the predominant full-length receptor, while the smaller band likely represents phosphorylated trkB receptors that have been previously described (Kang et al., 1997). Similar to our findings with trkB mRNA, our data demonstrated that estrogen does not regulate the level of trkB protein expression in the developing rat hippocampus. We did not observe any significant differences between intact males and castrated males given estrogen or vehicle. Also, consistent with our finding concerning trkB mRNA, we did not observe any developmental changes in trkB protein levels. These results suggest that estrogen is not acting by modulating trkB, but rather on BDNF directly.

Figure 29 Legend: trkB protein levels in the hippocampus of the developing male rat following castration and estrogen replacement. trkB levels were measured using Western blot analysis. (A) An immunoreactive doublet of approximately 145kDa was detected by Western blotting of hippocampal protein samples from intact, gonadectomized (GDX) and estrogen treated males during various stages of postnatal development. Males were GDX on postnatal day 0 and immediately treated with a single injection of oil vehicle or estradiol benzoate. (B) Following Western blot analysis of trkB and actin in the developing hippocampus of intact and hormone manipulated males, mean optical densities were determined of immunoreactive bands. Background levels were subtracted from these measurements and a ratio of trkB/actin was determined to give a mean net density for each age. Each bar represents the mean ± S.E.M. of three independent experiments. The levels of trkB expression following GDX were not significantly greater than the intact, or GDX + E treatment groups ($p > 0.68$).

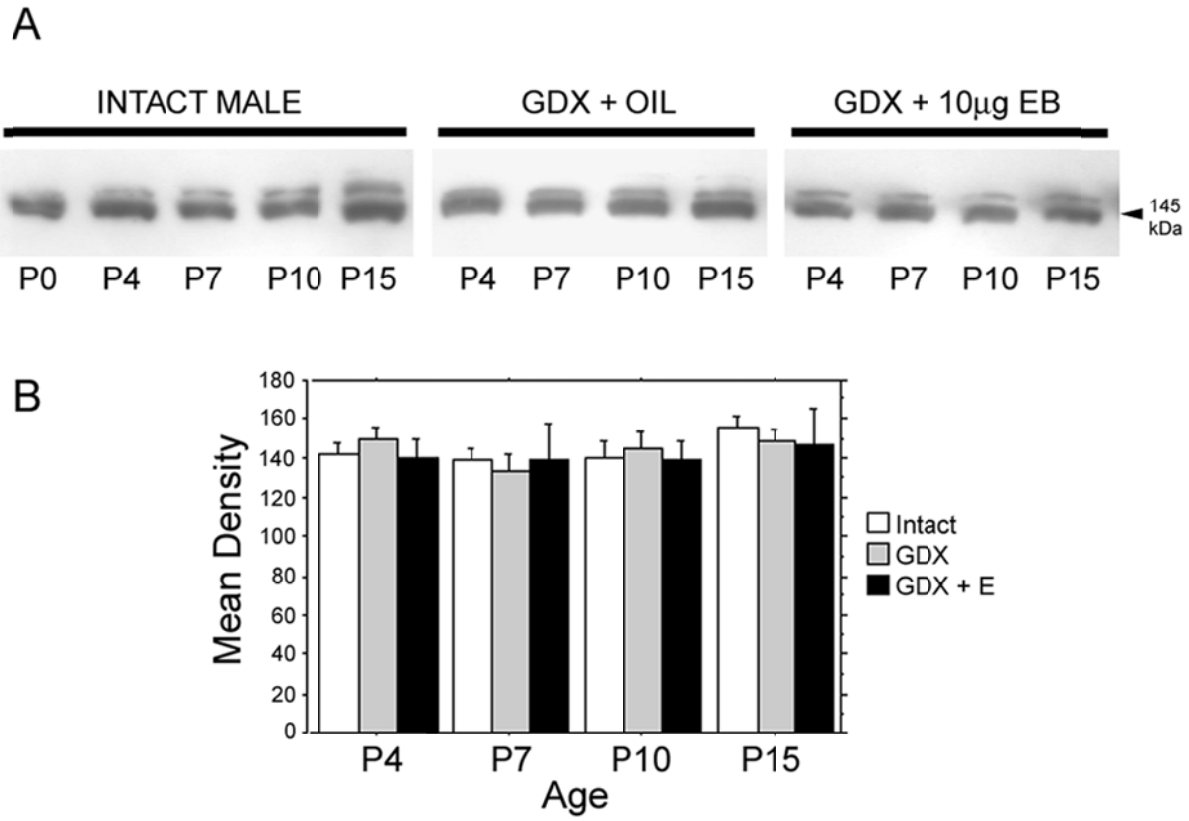

A
INTACT MALE
GDX + OIL
GDX + 10µg EB
145 kDa
P0 P4 P7 P10 P15
P4 P7 P10 P15
P4 P7 P10 P15
B
Mean Density
180
160
140
120
100
80
60
40
20
0
P4 P7 P10 P15
Age
Intact
GDX
GDX + E

*Figure 29*

## *Discussion*

In this study we examined the effect of early steroid environment on BDNF gene expression and demonstrated that neonatal gonadectomy and subsequent estrogen replacement regulates BDNF mRNA and protein expression in the developing rat hippocampus. A quantitative analysis of BDNF mRNA levels in the hippocampus following neonatal gonadectomy and subsequent estrogen replacement revealed a significantly increase in BDNF mRNA from postnatal days 4-10. Surprisingly, estrogen induced increases in BDNF mRNA were accompanied by a decrease in BDNF protein levels. However, there was no effect on trkB mRNA or protein expression. Thus, during development, estrogen could influence neuronal differentiation through regulation of BDNF.

Estrogen and numerous growth factors, including the neurotrophins, are associated with neuronal differentiation and survival. Estrogen is important during brain development influencing the maturation of neural systems and affecting the sexual differentiation of brain structures and functions. Estrogen affects both hypothalamic and hippocampal neuronal physiology and plasticity and promotes growth of the developing nervous system (Arnold and Gorski, 1984; Toran-Allerand, 1984). Unfortunately, the mechanisms by which estrogen affects the developing hippocampus are ill-defined.

The neurotrophins play important roles in neuronal survival and differentiation (for review, see Thoenen, 1995) and promotes neuronal survival during development and after various brain insults (Lindvall et al., 1994; Tucker et al., 2001). Studies have shown that neuronal cultures established during the early stages of neurogenesis are supported

by BNDF, whereas older neurons survive with NGF treatment (Enokido et al., 1999). Additionally, BDNF has been shown to induce the formation of both excitatory and inhibitory synapses of embryonic hippocampal cells (Vicario-Abejon et al., 1998), providing evidence that BDNF is essential during development.

BDNF has been postulated to be an important signaling molecule in regulating synaptic strength and overall circuit activity in the adult. Accordingly, chronic treatment with BDNF potentiates neurotransmission in the hippocampus (Bolton et al., 2000). BDNF and its receptor trkB regulate both short-term synaptic functions and long-term potentiation (LTP; McAllister et al., 1999). Estrogen has similarly been shown to enhance LTP (Warren et al., 1995; Foy et al., 1999; Good et al., 1999) and may regulate neural processes underlying learning and memory. The mechanisms by which estrogen modulates LTP are essentially unknown, but one possibility could be through interactions with the BDNF gene.

The co-localization of estrogen receptors with the neurotrophin receptors, $p75^{NGF}$, *trk*A and *trk*B, in the adult basal forebrain established an interaction between estrogen and the neurotrophins (Toran-Allerand et al., 1992; Miranda et al., 1993a). Subsequent work demonstrated the presence of an estrogen response element in the BDNF gene (Sohrabji et al., 1995) providing a direct link between estrogen and BDNF. Moreover, estrogen regulates BDNF gene expression in a developmental stage- and brain region-specific manner (Jezierski and Sohrabji, 2000), and this may play important roles in region and stage specific regulation of brain development. Similar to our findings in neonates, it has recently been demonstrated that estrogen significantly affects BDNF

mRNA and protein levels in a bi-directional manner within the adult rat hippocampus (Gibbs, 1999). Furthermore, other published reports have shown that estrogen (Liu et al., 2001) and phytoestrogens (Pan et al., 1999) significantly upregulate BDNF mRNA levels in adult mammals.

In addition to its effects on BDNF, estrogen has been shown to influence the expression of neurotrophin receptors. For instance, estrogen up-regulates mRNA for the NGF receptor, trkA (Sohrabji et al., 1994a; Sohrabji et al., 1994b), and increases trkB in the olfactory bulb (Jezierski and Sohrabji, 2000). However, consistent with the study of Gibbs (1998) in the adult hippocampus, we did not observe an effect of estrogen on trkB mRNA or protein during development. It is possible that estrogen regulates the trk receptors differently depending on the brain region or developmental period examined. Additional studies are needed to examine these differences.

Neurotrophic factors are assumed to provide trophic support via a target-derived, retrograde mechanism. Recent studies have demonstrated that BDNF can also act anterogradely similar to neurotransmitters (Smith et al., 1997; Altar and DiStefano, 1998). It is possible that BDNF produced in hippocampal neurons is transported to downstream targets, such as the basal forebrain and entorhinal cortex, and this transport could be influenced by estrogen as has been suggested for other brain regions (Jezierski and Sohrabji, 2000). This could account for the increased BDNF mRNA and decreased protein levels following estrogen treatment. This conclusion is supported by a recent report demonstrating an estrogen induced increase in BNDF levels in hippocampal targets (Liu et al., 2001). Alternatively, differential changes in turnover rates for BDNF

mRNA and protein or increases in BDNF levels following neonatal GDX due to retrograde uptake and storage from non-hippocampal sites could explain the differences. Estrogen may also act to regulate BDNF expression in different brain regions, or periods of development. For instance, Jezierski and Sohrabji (2000) recently demonstrated that estrogen increased BDNF levels in the olfactory bulb and DBB, but decreased them in the cingulate cortex. It appears that estrogen's effects on BDNF are complex and likely dependent upon the brain region or age examined.

Similar to our results *in vivo*, Murphy et al. (1998) showed that estrogen acts to down-regulate BDNF levels in hippocampal neurons grown *in vitro*. These authors suggested that estrogen acts through GABAergic interneurons to regulate BDNF expression. This was based on the findings of Weiland et al., (1997) showing ERα in interneurons of the adult rat. However, we have recently shown that during development, the highest concentration of ERα is in CA1 and CA3 pyramidal neurons, and double-labeling experiments demonstrated these cells did not express GABA (present findings and Solum and Handa, 2001). Based on our current findings showing that ERα is co-localized with BDNF in hippocampal pyramidal neurons and studies showing that BDNF is not expressed in interneurons (Pascual et al., 1999) we conclude that there is a direct action of ER on the BDNF gene in pyramidal cells. Even so, it is possible that estrogen regulates the expression of BDNF indirectly through a step involving other transcription regulating factors. For instance, Murphy et al. (1998) have suggested that BNDF levels could be regulated by CREB phosphorylation. Even so, this is consistent with our

findings, as estrogen has been shown to enhance CREB expression in the hippocampus (Panickar et al., 1997).

The discovery of ERβ (Kuiper et al., 1996) raised the possibility that some of estrogen's effects could be mediated by this receptor. While it has been shown that ERβ mRNA exist in the adult hippocampus (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001), ERβ mRNA does not appear to be translated at significant levels (Shughrue and Merchenthaler, 2001). Although ERβ has been described in the developing mouse hippocampus (Ivanova and Beyer, 2000), it has not been demonstrated whether ERβ protein exists during development. Here we have shown that ERβ is indeed found at low levels in the developing hippocampus but it is not co-localized with BDNF, suggesting the predominant interaction is through ERα. Studies examining splice variants of ERβ mRNA have suggested that the predominant form in the hippocampus is a variant that does not bind estrogen (Price et al., 2001). Since the two estrogen receptor types can activate different signaling pathways (Webb et al., 1999), it is possible these receptor types may differentially influence the extent and direction of BDNF expression. While the mechanism underlying estrogen effects on BDNF gene expression remains unclear, differences in ERα and ERβ signaling mechanisms (Jones et al., 1999), and regional expression patterns (Shughrue et al., 1997) may help explain these differences.

Our studies used real-time PCR to analyze the expression of BDNF and trkB mRNA following neonatal gonadectomy. Using a traditional thermal cycler, which relies on end-point quantitation, there is theoretically a quantitative relationship between the

amount of starting target sequence and amount of product following amplification. Unfortunately, quantitation relies on carefully titrating the product to the linear part of the amplification curve which is, in reality, a very narrow range. Furthermore, replicate reactions often yield different amounts of PCR product unless elaborate, time-consuming controls are used. Real-time PCR has reduced the variability and expanded the linear range for quantitation by monitoring the product amount after each cycle (Wittwer et al., 1997). Therefore, the optimum cycle number does not need to be determined empirically. This technique has been proven to be a sensitive and quantitative way to assess gene expression in normal development and during pathophysiological conditions (Li and Wang, 2000; Wang et al., 2000) as well as to quantify steroid hormone receptor mRNA levels (Latil et al., 2001).

In summary, previous work in this laboratory has shown that in the neonatal hippocampus, ERα is highly expressed during development. In this study, we have demonstrated that estrogen regulates the expression of BDNF mRNA and protein in the developing rat hippocampus. It is possible then, estrogen, by binding the ERα receptor, directly alters BDNF gene expression. These findings may contribute to our understanding of the mechanisms by which steroid hormones influence the differentiation of developing neurons.

# CHAPTER 6

# DEVELOPMENTAL EXPRESSION OF THE p160 COACTIVATORS IN THE RAT BRAIN


## *Abstract*

During development, steroid hormones permanently impact the morphology and physiology of neurons. These effects are mediated by nuclear hormone receptors which, following ligand binding, regulate gene transcription and neuronal function. Additionally, The control of target gene expression by steroid hormone receptors requires the recruitment of multiple cofactors for efficient transcriptional activity. Here we describe the developmental expression of messenger RNA for the p160 steroid receptor coactivators (SRCs), including SRC-1/N-CoA1, SRC-2/GRIP-1/TIF2 and SRC-3/AIB1/p/CIP in several regions of the rat brain using quantitative real-time PCR. Messenger RNA expression levels were quantitated in the medial basal hypothalamus, prefrontal cortex and the CA1 and CA3 regions of the hippocampus from postnatal (P) day 0, 4, 7, 10, 15, 20 and adult rats. Our results demonstrate that these coactivators show significantly different levels of mRNA expression, with SRC-1 generally expressed at the highest levels, and SRC-2 at the lowest in all brain regions examined. The level of expression for SRC-3 was more variable depending on the brain region examined, but

was usually less than SRC-1. The developmental timecourse of mRNA expression was also variable for these coactivators. SRC-1 was expressed at high levels at all ages examined. SRC-2 and 3, in contrast, showed changes in expression patterns over the course of development. For example, in the hippocampus, the levels of SRC-2 were undetectable at P0, and increased transiently to their highest levels at P15. SRC-3, on the other hand, was generally expressed at low levels early in development, with a gradual increase in expression over development. This pattern was similar for all brain regions examined. These findings suggest that the p160 coactivators play differening roles during brain development to enhance transcriptional activation of steroid hormone receptors.

## *Introduction*

Steroid hormones influence transcriptional activation through specific nuclear receptors (NRs), including the estrogen receptor (ER) and progesterone receptor (PR). These receptors function as ligand-activated transcription factors and work in conjunction with a variety of coregulatory proteins. Ligand activated nuclear hormone receptors bind to specific DNA sequences in the enhancer region of hormone-responsive genes, termed hormone response elements, where they serve to stimulate or repress transcription of hormone responsive genes. Nuclear hormone coactivators and corepressors are molecules that form a bridge (either function or physical) between the receptor and the RNA II polymerase holoenzyme complex. Coregulatory proteins include members of the p160 family of steroid receptor coactivators. Coactivator proteins are often part of

multiprotein complexes that enhance the activity of NRs and other transcription factors (Horwitz et al., 1996; McKenna et al., 1999a; Glass and Rosenfeld, 2000). The recruitment of coactivator proteins to the basal transcription machinery by steroid hormone receptors when complexed with their respective ligands is correlated with transcriptional activity. While very little attention has been given to the role of coactivator proteins in the brain, some studies have demonstrated that some coactivator proteins are located in neurons (Martinez de Arrieta et al., 2000; Meijer et al., 2000; Ogawa et al., 2001), and may influence the function of receptors in brain functions such as the sexual differentiation of the brain (Auger et al., 2000).

Over 30 potential coactivator proteins have been identified based on their ability to bind various receptor domains and to alter the transcriptional activity of NRs (McKenna et al., 1999a; Glass and Rosenfeld, 2000). These coactivator proteins function as signaling intermediates between the receptors and the general transcriptional machinery to affect gene transcription (Horwitz et al., 1996; Katzenellenbogen et al., 1996). Distinct classes of ligand-dependent transcriptional cofactors have been described, including CBP/p300 and the p160 family (Xu et al., 1999). At present, the p160 family contains three members including SRC-1/N-CoA1 (Onate et al., 1995; Kamei et al., 1996), SRC-2/GRIP-1/TIF2 (Hong et al., 1996; Voegel et al., 1996; Torchia et al., 1997) and SRC-3/AIB1/p/CIP/RAC3 (Li et al., 1997a; Torchia et al., 1997; Suen et al., 1998). The p160 family is defined by an overall sequence similarity of 40% between the three proteins, distinguishing its members from other coregulator classes. The highest degree of homology is in the N-terminal domain, where the basic helix-loop-helix

(bHLH) domain exhibits a high degree of similarity. This bHLH domain is responsible for mediating protein-protein interactions with other transcriptional machinery, including CBP/p300 and RNA polymerase II.

How coactivator proteins function to enhance transcriptional activation is currently speculative, but attention centers on their interactions with the basal transcription machinery and on their associated histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Chen et al., 1997). For example, coactivator proteins play a role in chromatin remodeling, a process that allows the binding of other transcription factors (Kadonaga, 1998). Subsequently, coactivators interact with transcription machinery, such as RNA polymerase II, to transcribe target genes. Thus, the specific recruitment of a protein with histone acetyltransferase activity to a gene promotor region may play a critical role in transcriptional activation by a hormone receptor. Moreover, in the absence of coactivator proteins, significant transactivation of steroid receptors is unlikely to occur.

Steroid receptor coactivator 1 (SRC-1; also N-CoA1) is a 160kDa protein that has been shown to enhance transcription mediated by steroid hormone receptors, including PR and ER (Onate et al., 1995) in addition to functioning with the coactivator CBP/p300 (Kamei et al., 1996; Smith et al., 1996). SRC-1 enhances the activity of ligands that have pure agonist activity (Llopis et al., 2000) as well as those with mixed agonist/antagonist properties (Katzenellenbogen et al., 1996). For instance, 17b-estradiol has been shown to create a strong association between ERa and SRC-1, and this interaction is disrupted by the antagonist ICI 182,780 (Stenoien et al., 2000). It has been demonstrated that

peripheral steroid target organs of mice containing a targeted disruption of the SRC-1 gene have a decreased response to steroid hormones such as estrogen and progesterone (Xu et al., 1998), demonstrating its importance in steroid hormone receptor action. Additionally, the distribution of SRC-1 has been shown to be extensive in both peripheral (Onate et al., 1995; Misiti et al., 1998) and nervous tissues (Martinez de Arrieta et al., 2000; Meijer et al., 2000; Ogawa et al., 2001), although little is known about its expression during development.

Steroid receptor coactivator 2 (SRC-2; also GRIP1/TIF2) has significant homology to SRC-1 (Hong et al., 1996; Voegel et al., 1996) and mediates transcriptional activation by members of the steroid receptor superfamily. SRC-2 interacts in a ligand-regulated manner with the hormone binding domain of the glucocorticoid receptor (GR) and androgen receptor (AR) as well as ER (Hong et al., 1996). While very few studies have examined the expression of SRC-2 in brain tissue, its distribution appears to be much more restricted than SRC-1 (Xu et al., 1998; Meijer et al., 2000).

Like the other two p160 coactivator family members, steroid receptor coactivator 3 (SRC-3; also AIB1/p/CIP/RAC3) interacts with a wide variety of nuclear receptors in a ligand dependent manner (Anzick et al., 1997). Similar to SRC-2, SRC-3, has a variable expression pattern in peripheral tissues, and its distribution appears to be more restricted than SRC-1 in brain (Suen et al., 1998; Xu et al., 2000). SRC-3 has the ability to enhance the transcription mediated by the progesterone receptor (PR), and selectively enhances the transcriptional activity of the ERα subtype over ERβ (Suen et al., 1998). The

receptor selectivity observed with SRC-3 may subsequently underlie regional or tissue differences in receptor activation.

In this study, we examined the developmental expression of mRNA for steroid receptor coactivators in multiple regions of the rat brain using quantitative real-time PCR. We show that mRNAs for p160 steroid receptor coactivators are variably expressed in different brain regions, and that the developmental time course for their expression is also variable depending on the brain region examined. Thus, the differential expression of these coactivators may underlie the diversity of steroid receptor mediated signals in the developing brain.

## *Material and Methods*

*Animals*. Neonatal male and female rats of various ages taken from timed-pregnant Sprague-Dawley females and male and female adult rats were obtained from Charles River Laboratories (Portage, MI). Animals were housed under a 12/12 h light dark cycle (lights on at 07.00 h) with food and water available *ad libitum*. Upon parturition, litters were sexed and thinned to 8 pups (4 males and 4 females). Adult females were sacrificed at 3-4 months of age. All animal protocols were previously approved by the Animal Care and Use Committee at Colorado State University.

*Total RNA isolation and reverse transcription.* Neonatal rat pups were decapitated, and the brains quickly removed. Medial basal hypothalamus, cortex, and hippocampal regions CA1 and CA3 were then rapidly dissected. Total RNA was isolated according to the protocol of Chomczynski and Sacchi (1987). Briefly, brain tissue was

homogenized in 250µl of GIT buffer (4M guanidinium isothiocyanate/25mM NaCitrate, pH 7.0/0.5%sarcosyl/0.1M β-mercaptoethanol) on ice. Subsequently, 25µl of 2M NaOAc (pH 4.0), 250µl of buffer-saturated phenol (pH 4.3) and 50µl of chloroform/isoamyl alcohol (49:1) was added and the mixture vortexed. The samples were then centrifuged at 14,000 x g for 20 minutes at 4ºC. The aqueous phase was recovered and the RNA was ethanol precipitated. The resulting RNA was washed with ice-cold 70% ethanol, and reconstituted in 50µl RNAse free water. The RNA content was measured with a spectrophotometer and only those samples with a 260/280 ratio between 1.6 and 2.0 were used.

2 µg of total RNA was reverse transcribed with Superscript II reverse transcriptase (RT; Gibco/BRL, Rockville, MD) using oligo-dT primers, deoxyribonucleotides (dNTPs; 100 mM each), 1st strand buffer (100 mM Tris-Cl/900 mM KCl/1 mM $MgCl_2$) and 2.5 mM dithiothreitol (DTT). The reaction was carried out at room temperature for 10 minutes followed by 50 minutes at 42ºC. The RT was then denatured at 95ºC for 10 minutes and stored at –80ºC until used.

*Real-time quantitative polymerase chain reaction (PCR)*. Real-time quantitative PCR was performed using the LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (Solum and Handa, 2001b). In this system, A highly specific double-stranded (ds) DNA binding dye, SYBR Green I (Molecular Probes, Eugene, OR), which only fluoresces when bound to dsDNA, is used to determine the concentration of amplified products. SYBR Green I binds to the minor groove of

dsDNA and fluorescence is greatly enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending the amount of dsDNA that is present. The 530 nm fluorescence is recorded at the end of the elongation phase, and increasing amounts of PCR product are monitored from cycle to cycle. By comparing the amount of unknown cDNA to a curve of amounts of a given cDNA amplified concurrently, real-time PCR eliminates the need for competitive in-tube standards with identical primer sets as targets (Morrison et al., 1998).

To prevent nonspecific amplification we used hot-start PCR with dNTPs, specific primers, PCR buffer (100 mM Tris-Cl, 1.5 mM $MgCl_2$), 0.5 units Taq polymerase, 0.5 units Taq antibody (Gibco/BRL) and 2 μl of 10X stock SYBR Green I. Specific primers were as follows: SRC-1 (Genbank accession #NM 010881) forward primer position 3433, reverse primer position, 3954; SRC-2 (Genbank accession #AF136943) forward primer 3179, reverse primer 3596 and SRC-3 (Genbank accession #AF322224) forward primer position 167, reverse primer position 500. Primers were developed using OLIGO software (v. 6.51; Molecular Biology Insights, Cascade, CO). All samples were amplified at 40 cycles, which is approximately five to ten cycles beyond the beginning of the linear phase of amplification. Specifically, an initial melting step was done at 95ºC for 2 minutes followed for 40 cycles of 95ºC melting step for 1s, an annealing step (60ºC for SRC-1 and 64ºC for SRC-2 and 3) for 5s, and a 72ºC elongation step (21s for SRC-1, 16s for SRC-2 and13s for SRC-3). In all experiments, samples containing no template were included to serve as negative controls.

*Construction of cDNA standard curves*. To determine the absolute concentration of the target transcript, conventional PCR for SRC1-3 was used to generate a cDNA. The amplified cDNA was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) according to the manufacture's directions. The purified PCR products were serially diluted at a range of 30ng-30fg and this curve was run in duplicate alongside the unknown samples.

*mRNA quantitative analysis*. Following real-time PCR, the absolute concentration of mRNA in each sample was determined by analysis with LightCycler Data Analysis (LCDA) software. This software plots a standard curve of the crossing line intercepts of the standards vs. the known concentrations of these standards. The crossing line intercept is parallel to the x-axis on a graph of fluorescence intensity vs. cycle number and occurs at the point where template amplification enters the logarithmic phase of the curve. Samples with a higher concentration of starting material enter the logarithmic phase earlier than samples with a lower concentration of starting material and therefore have a smaller crossing point value. The crossing line intercept of an unknown sample is subsequently compared with the standard curve to generate a quantitative amount of starting material. In each case, the point at which the crossing line intercepts the log-linear region of each curve is used to generate the concentration of that sample.

*Statistical analyses*. All analyses were performed using analysis of variance (ANOVA, StatView, SAS Institute Inc.). Significant values were subsequently verified with the Tukey-Kramer post hoc analysis.

## *Results*

Expression of steroid receptor coactivator mRNA in the hypothalamus

We first examined the expression of messenger RNA for the p160 steroid receptor coactivators (SRCs 1-3) in the medial basal hypothalamus of intact female rats during development from postnatal day 0 through adulthood (Fig 30). Steroid receptor coactivator 1 (SRC-1) mRNA is expressed at relatively high levels in the hypothalamus, and these levels do not change significantly from birth to adulthood. The coactivator SRC-2, on the other hand, has the lowest level of expression of the three coactivators examined. Moreover, the level of expression did not change significantly throughout development. The last coactivator examined, SRC-3, maintained a level of expression that while only slightly and insignificantly greater that SRC-2 at most ages, was much less than SRC-1 at all time points. Even so, the expression of SRC-3 mRNA levels showed a trend toward gradually increasing concentrations, such that by adulthood the levels were significantly greater that those at birth ($p < 0.03$). The results of these studies demonstrate that while significant differences in the levels of mRNA expression for these coactivators exist in the medial basal hypothalamus, they are all present during postnatal development. Moreover, the levels of expression for these coactivators are high enough to suggest they play some role in the transcriptional modulation of steroid responsive genes in the developing hypothalamus.

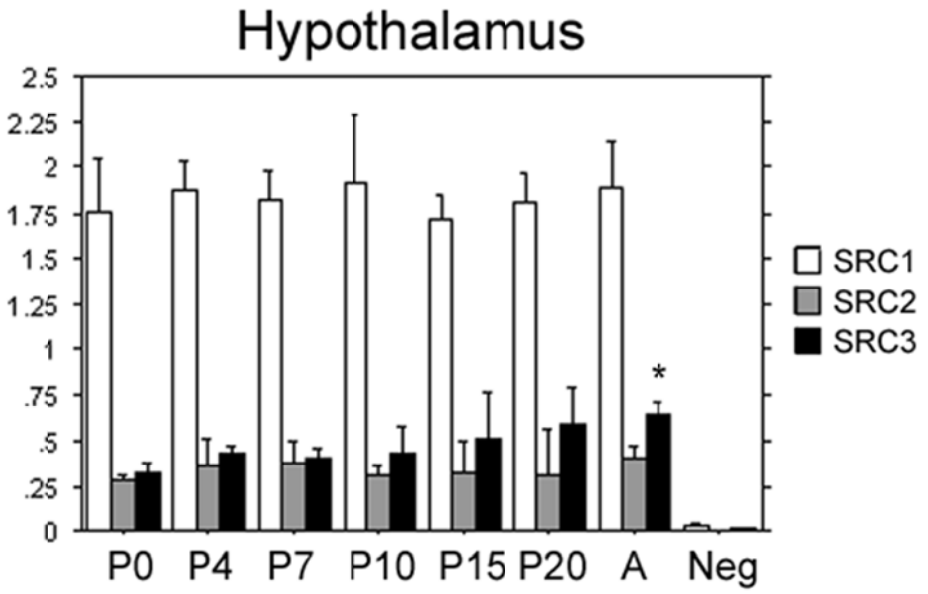


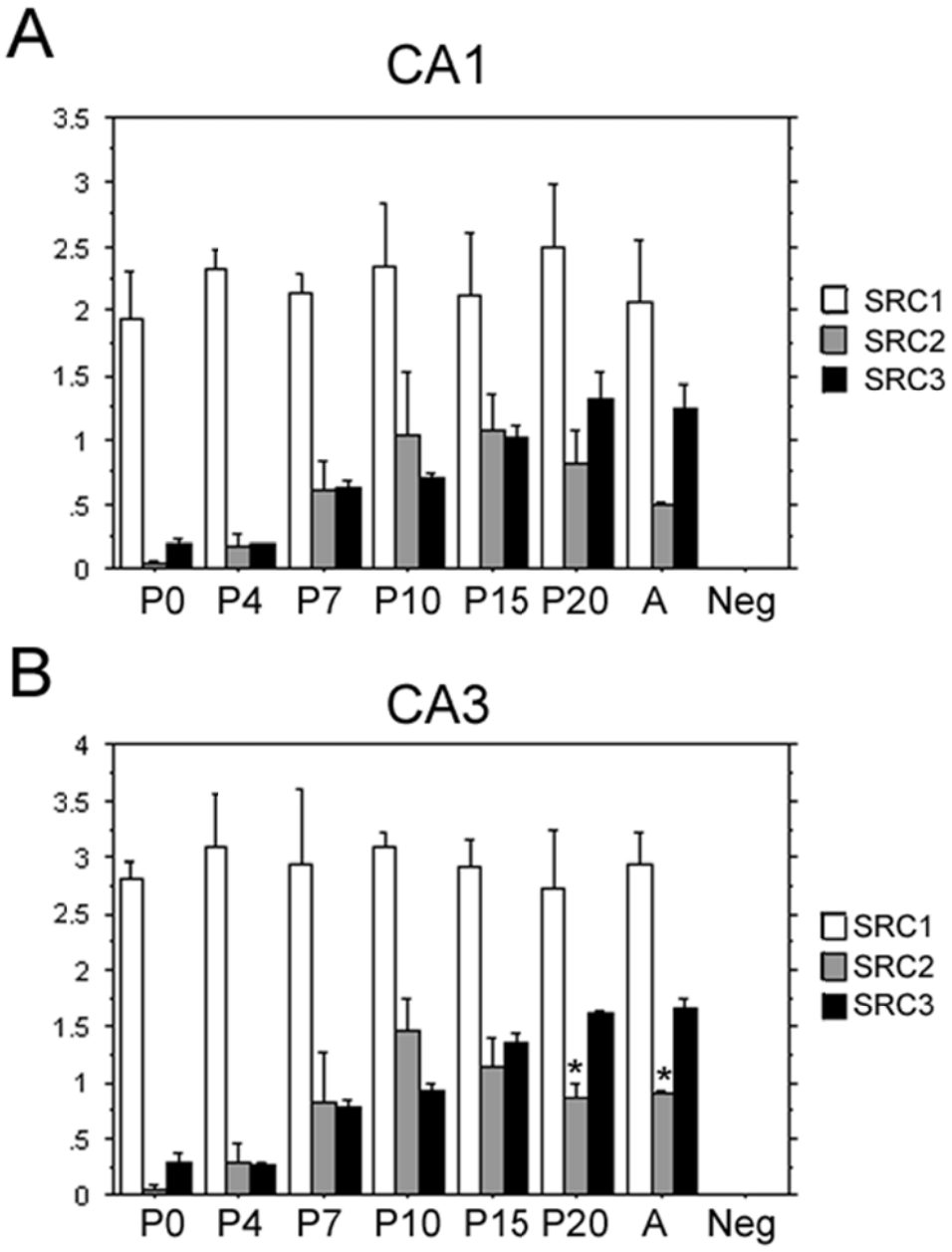

*Figure 30*

Figure 30 Legend: Expression of p160 steroid receptor coactivator mRNA in the hypothalamus. Real-time PCR was used to examine the expression of steroid receptor coactivator (SRC) 1-3 messenger RNA in the hypothalamus during development. All values are reported in picograms of cDNA. Each bar represents the mean ± S.E.M. of three independent experiments. No significant changes were observed for SRC-1 ($p > 0.88$) or SRC-2 ($p > 0.59$) over development. Expression of SRC-2 gradually increased from birth, such that by adulthood, the levels were significantly greater than P0 ($p < 0.03$). (*) Significantly greater than P0.

Expression of p160 steroid receptor coactivator mRNA in the hippocampus

The presence of p160 coactivators in the hypothalamus during development and adulthood subsequently raised additional questions concerning their expression in other brain regions. Therefore, we examined the expression of messenger RNA for steroid receptor coactivators (SRCs 1-3) in hippocampal subregions CA1 and CA3 of intact female rats during normal development (Fig 31). Similar to our findings in the hypothalamus, SRC-1 expression levels in the hippocampus were very high during development in both CA1 and CA3, and these levels did not change with increasing age from birth through adulthood. In contrast to SRC-1, the pattern of SRC-2 mRNA expression showed a much different pattern during development. At birth, the levels of SRC-2 mRNA were not detectable. Beginning on postnatal day 4, the levels increased to peak by P10, then declined gradually to lower levels which were observed in adult animals. The decline in expression from P10 to adulthood was much more pronounced in the CA3 subregion. In fact, in the CA1 subregion, the levels at postnatal day 15 and 20 were not different from those at P10, but in the CA3 region the levels of SRC-2 were significantly reduced by P20. SRC-3 mRNA levels were barely detected on P0, but in each subsequent age examined, the levels of expression increased. This remained the case until P20, at which point mRNA levels were not different from adults. The developmental changes in mRNA levels for SRC-3 was essentially the same in both the CA1 and CA3 hippocampal subregions.

Figure 31 Legend: Expression of p160 steroid receptor coactivator mRNA in the hippocampus. Quantitative real-time PCR was used to examine the expression of SRC1-3 mRNA in the CA1 (A) and CA3 (B) hippocampal subregions during development. All values are reported in picograms of cDNA. Each bar represents the mean ± S.E.M. of three independent experiments. No significant changes were observed for SRC-1 over development ($p > 0.76$). In both the CA1 and CA3 subregions, the level of expression for SRC-2 increased from P0 to peak at P10. These levels decreased, and in the CA3 subregion, the levels in adults were significantly less than at P10 ($P < 0.01$). The levels of SRC-3 mRNA were very low shortly after birth, and increased gradually over development to reach their highest in adulthood. In fact beginning on P7 the levels of SRC-3 mRNA were significantly greater than at birth. Unlike SRC-2, the levels for SRC-3 did not drop after the second postnatal week. (*) Significantly less than P10.


A
CA1
3.5
3
2.5
2
1.5
1
.5
0
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P10
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SRC1
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SRC3
B
CA3
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3.5
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P10
P15
P20
A
Neg
SRC1
SRC2
SRC3

*Figure 31*

Expression of steroid receptor coactivator mRNA in the prefrontal cortex

In the cortex, the levels of expression for SRC-1 followed a pattern of expression similar to that of the hypothalamus and hippocampus with one notable exception. That is, while the level of expression of SRC-1 did not change over development in the cortex, they were much greater than the hypothalamus and hippocampus at all ages examined (Fig 32). In fact, at some ages the levels of cDNA were nearly double of those observed in the other brain regions examined. The physiological significance of this finding is unknown. The levels of SRC-2 were significantly less than those for SRC-1, and slightly less than those of SRC-3 at most ages examined. These overall differences in expression were maintained throughout development. Moreover, In contrast to the hypothalamus and hippocampus no developmental changes were observed for any of p160 coactivators in the cortex. This was a surprising finding considering that many substantial changes are taking place in the cortex during the developmental period examined. Nevertheless, the SRCs are expressed at levels high enough to suggest that, if translated, they would play important roles in steroid hormone action.

Localization of SRC-1 in the developing rat brain

Next, we used immunocytochemistry to determine whether SRC-1 expression was similar to that of its messenger RNA. Our results demonstrated a pattern of expression for SRC-1 that was consistent with that of its messenger RNA (Fig. 33). Analysis of the distribution of SRC-2 and SRC-3 was hindered by the inability of currently available antibodies to recognize these proteins in brain tissue. At all ages

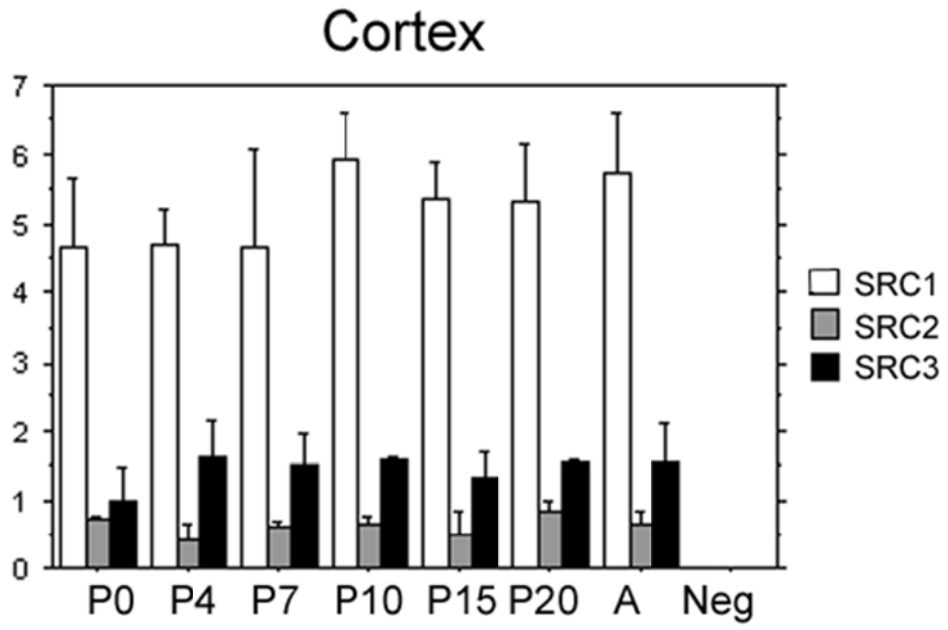


*Figure 32*

Figure 32 Legend: Expression of mRNA for the p160 coactivators in the developing cortex. Real-time PCR was used to examine the expression of steroid receptor coactivator (SRC) 1-3 messenger RNA in the cortex during development. All values are reported in picograms of cDNA. Each bar represents the mean ± S.E.M. of three independent experiments. No significant changes were observed for SRC-1 ($p > 0.49$), SRC-2 ($p > 0.81$) or SRC-3 ($p > 0.88$) over development.

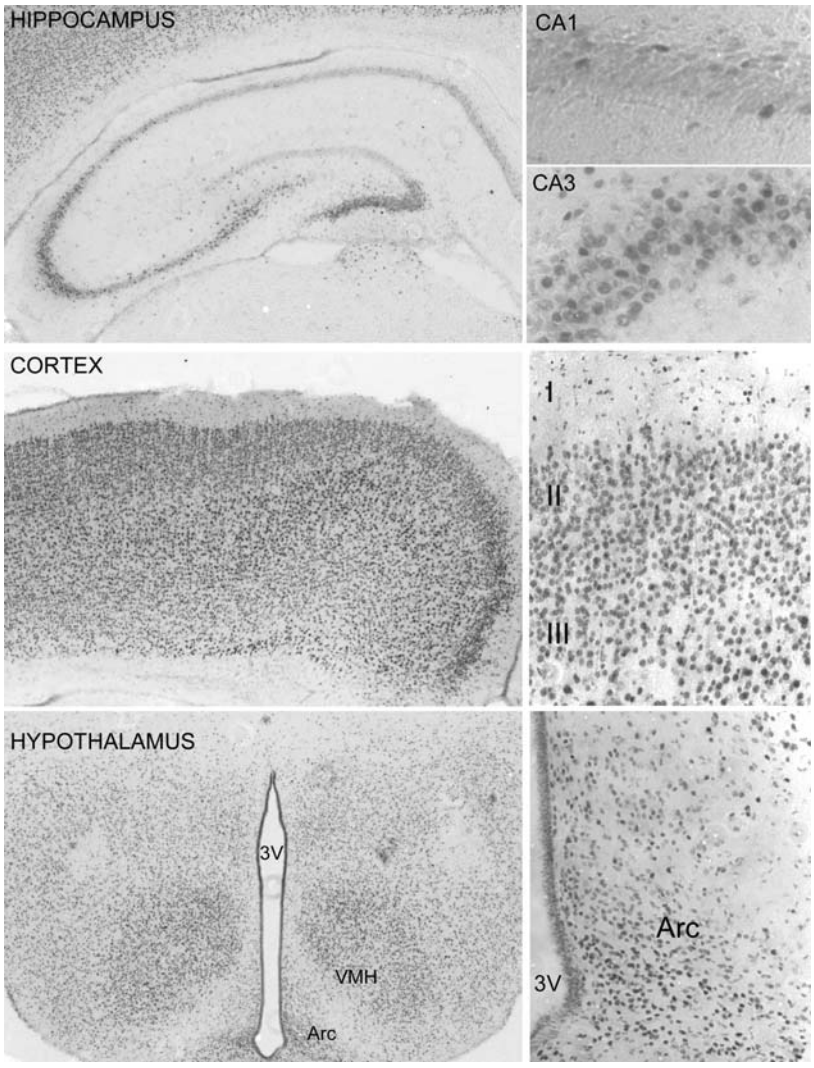


Figure 33

Figure 33 Legend: Expression of SRC-1 in the developing brain. SRC-1 is highly expressed in the hippocampus, hypothalamus and cortex of the developing rat. In the hippocampus, the majority of staining is observed in the CA3 region, with CA1 and dentate gyrus lower. In the cortex, layers 2-6 are heavily stailed while in the hypothalamus a large number of cells in the arcuate and ventral medial nuclei are immunopositive.

examined, SRC-1 immunoreactivity was concentrated to the nucleus of cells in the hypothalamus, hippocampus and cortex. Moreover, in these studies we never observed cytoplasmic staining for this coactivator, and within the nucleus, the intensity of staining was usually quite high. In the hypothalamus, SRC-1 immunoreactivity was moderately expressed, with the arcuate and ventral medial nuclei consistently stained darkly. The supraoptic nucleus was also notably immunopositive. In the hippocampus, immunoreactivity was very strong in stratum pyramidale of both CA1 and CA3 subregions. SRC-1 immunoreactivity in the cortex was most intense in layers 4-6, but cells in layers 2 and 3 were also immunopositive. Similar to our findings on the expression of SRC-1 mRNA in the developing brain, the levels of immunoreactivity did not change drastically with increasing age. In general, the number and intensity of immunoreactive cells was high beginning on P0, and in each subsequent age examined, the number and intensity of SRC-1 immunoreactive cells remained high.

## *Discussion*

Steroid hormones, including estrogen, exert important roles in the development of the central nervous system by acting in cell and temporally-specific ways. However, the estrogen receptors and other NRs are much more than simple switches that translate the presence of hormones into changes in gene expression. The responses of steroid hormones depend on multiple cofactors, such nuclear receptor (NR) coactivator proteins. Following a quantitative analysis of messenger RNA levels for the p160 coactivators in

the developing rat brain, we observed a unique developmental and regional pattern of expression for these coactivators. These distinct patterns of expression for the p160 coactivators may subsequently underlie tissue or ontogenetic differences in the ability of these proteins to influence transcriptional activation in the brain. Additionally, we have demonstrated that like its mRNA, SRC-1 protein is highly expressed in the hippocampus, and is colocalized with ERα in neurons from this brain region. SRC-1 is also highly expressed in the developing cortex, and to a lesser extent in the medial basal hypothalamus. To our knowledge, this is the first report to describe the developmental expression of the p160 coactivator messenger RNAs in brain tissue.

The nuclear receptors are transcriptional regulators that activate gene transcription upon binding of their respective ligands. While there is considerable evidence that nuclear receptors can contact some of the basal factors of the preinitiation complex directly, without the need for intermediary coregulatory proteins, evidence for additional factors is supported by observations that different classes of receptors can interfere with one another's transcriptional activity by squelching limiting factors that are not components of the basal transcription machinery (Meyer et al., 1989). Studies aimed at understanding the mechanism by which NRs regulate target gene transcription have discovered several novel coactivators that increase the ability of the receptors to activate transcription (Horwitz et al., 1996). It has been suggested that these cofactors act as a bridging apparatus between the receptor and the transcriptional machinery (Chiba et al., 2000; Glass and Rosenfeld, 2000). Additionally, ligand dependent recruitment of p160 coactivators, in concert with other factors such as CBP and p300, bring acetyl transferase

activity to NR complexes. The coactivator proteins, then, coordinate the interactions of steroid receptors, their ligands, and transcriptional machinery to initiate gene transcription.

In this report, we find expression patterns that are specific for each p160 coactivator, suggesting different roles for these three genes and differences in regional sensitivity for steroid signaling in the developing brain. Such differences in expression levels might indicate cell specificity of NR-mediated transcriptional regulation, and might partially explain how the same gene can be regulated differentially in different cell types (Jain et al., 1998). This idea is further supported by the SRC-1 and SRC-2 knock out mice which demonstrate distinct NR coactivators may have specific physiological functions based on the different phenotypes these mice exhibit (Xu et al., 1998; Xu et al., 2000). While mice lacking p160 coactivators are viable and grossly normal, SRC deletions resulted in steroid and thyroid hormone resistance, suggesting that such states could be manifestations of dysfunctional NR coactivators. It appears then, that the p160 coactivators play a more important role in the tissue specific effects of steroid hormones than just to provide a functional redundancy for the other class members.

While some coregulatory proteins function as coactivators for a variety of transcription factors, the p160 family, including the SRCs, appears to be restricted to the nuclear receptor family (Torchia et al., 1997; Korzus et al., 1998). Not only do SRCs enhance transactivation by steroid hormones, but also in certain circumstances, significant transactivation mediated by steroid hormones is not likely to occur in their absence (Llopis et al., 2000). For instance, studies with the estrogen receptor have

demonstrated that SRC-1 is required for transcriptional activation, and this activation is enhanced in a ligand dependent manner (Onate et al., 1995; McKenna et al., 1999b; Llopis et al., 2000).

The developing brain is a sensitive target for steroid hormones, which mediate several events and have lasting effects on adult physiology and behavior (Harris and Levine, 1962). Additionally, steroid hormones, including estrogen and thyroid hormone, act during a perinatal sensitive period of development to influence the development of brain structures (Hajos et al., 1973; McEwen, 1983).

Similar to our findings presented here, other studies have shown that the abundance of transcripts for SRC-1 is of the same order for many brain regions (Meijer et al., 2000). However, this finding may be specific to the brain region examined as another recent report demonstrated a developmental increase in the levels of SRC-1 mRNA levels in the cerebellum (Martinez de Arrieta et al., 2000), a brain region not examined in the present study.

In contrast to our results presented here, Meijer and colleagues (2000) reported that they were unable to detect SRC-2 mRNA in brain tissue. This is possibly due to the fact that Meijer et al. (2000) used *in situ* hybridization with end-labeled oligonucleotides, a technique with much less sensitivity than RT-PCR. This technique would be unlikely to detect small concentrations of mRNA such as what we have reported here for SRC-2 mRNA. Nevertheless, in experiments using adult mice, it was demonstrated that SRC-3 gene expression is restricted in a tissue and cell type-specific manner (Xu et al., 2000), supporting our findings with SRC-3 mRNA in the developing rat. The present findings,

taken together with earlier reports examining the distribution of p160 coactivators, underscore the variability associated with the expression patterns of these genes. Clearly, additional studies need to be conducted to elucidate the significance of the p160 coactivators in the developing brain.

In summary, p160 coactivator genes appear to be developmentally regulated in the rat brain, with each gene following a unique pattern of expression throughout development. SRC-1 was generally expressed at the highest levels in all brain regions examined, including the hippocampus, hypothalamus and cortex, with lower levels of SRC-3 and SRC-2 consistently observed. The presence of these genes in the developing brain suggest they play an important role in the coordination of transcriptional activation by steroid receptors.

# CHAPTER 7

# OVERALL DISCUSSION

In recent years, the knowledge of how estrogen influences mammalian brain functions and development has broadened substantially. In the adult brain, estrogen is not only involved in the neuroendocrine feedback regulation at the hypothalamic and pituitary level but also in the control of cognitive functions (Sherwin, 1997). More recently, estrogen was suggested to act as a neuroprotective factor for neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Henderson et al., 1996; Leranth et al., 2000). In addition to these regulatory and protective functions, estrogen plays an important role during development. After the demonstration that the estrogen-synthesizing enzyme aromatase (Harada and Yamada, 1992) and both nuclear estrogen receptors (Jakab et al., 2001 ; DonCarlos, 1996; Yokosuka et al., 1997; Ivanova and Beyer, 2000) are expressed in many brain regions during ontogeny, it was soon realized that estrogen modulates neuronal differentiation, notably by influencing cell survival and death, and synaptic plasticity of neurons. These effects were initially seen in the classical target area for estrogen, the hypothalamus, but successive studies revealed the neurotrophic potential of estrogen in other brain regions as well. One example of an extrahypothalamic brain region that is influenced by steroid hormones is the hippocampus. This brain region is involved with learning and memory tasks, cognition,

and stress responsivity, all of which show sexual dimorphisms (Williams et al., 1990; Williams and Meck, 1991; Kellogg, 1999; Lavenex et al., 2000). The mechanisms by which estrogen exerts its neurotrophic and neuroprotective roles are essentially unknown, but an attractive hypothesis involves an interaction with brain-derived neurotrophic factor (BDNF). BDNF has been shown to be important in both of these roles; that is, it is neuroprotective in adulthood, and neurotrophic during development (Barde, 1989; Davies, 1994). Although the exact mechanism by which estrogen interacts with BDNF is not known, there are several possibilities. One involves a direct interaction of wildtype estrogen receptors with the estrogen response element in the BDNF gene. This is the simplest and most likely possibility. Alternatively, estrogen's effects could be mediated by a δ3 variant of ERβ that doesn't bind efficiently to EREs, but does initiate transcription at AP-1 response elements. Alternatively, estrogen may be acting indirectly through second messenger systems such at the MAP kinase pathway. Finally, the effects of estrogen on BDNF could be mediated through protein-protein interactions with coactivator proteins such as the p160s and CBP/p300, which bind to the CREB response element in the BDNF gene promotor.

In the studies presented in this dissertation, some potential mechanisms by which estrogen influences the hippocampus during early postnatal development have been examined utilizing both *in vivo* and *in vitro* models. This project was conceived based on the data presented in chapter three which describes the developmental expression of estrogen receptor alpha in the hippocampus. Our findings suggested that estrogen might be acting very differently than had been described in the literature based on the location

of ERα we observed in the developing hippocampus. For instance, I demonstrated that during postnatal development, ERα was predominantly localized to pyramidal neurons of the CA1 and CA3 subregions of the hippocampus, and not restricted to spiny interneurons as had been previously described in adults (Weiland et al., 1997). Interestingly, it had been demonstrated in the adult that hippocampal pyramidal cells display a robust response to estrogen treatment and to fluctuations in ovarian hormones across the estrous cycle (Gould et al., 1990; Woolley and McEwen, 1992). Furthermore, during the second postnatal week, a time when we observe the highest concentration of ERα, the plasma levels of estradiol are also high (Meijs-Roelofs et al., 1973; Dohler and Wuttke, 1975). Therefore, our results were exciting considering the distribution of ERα we observed which coincided with the transient increase in plasma estrogen levels and suggested that estrogen might be acting directly on the excitatory principle cells of the hippocampus rather than through an indirect mechanism involving the inhibitory neurotransmitter γ-amino butyric acid.

The data presented in this dissertation also raise another interesting question concerning the time frame for the organizational effects of steroid hormones in different brain regions. Studies in the hypothalamus showed gonadal hormones influence sexual differentiation from embryonic day 18 through about the second week after birth (MacLusky and Naftolin, 1981), and these effects are likely mediated through the estrogen receptor. Our results, showing the peak of ERα expression at P10 in the hippocampus, which is relatively late in relationship to the “critical period” of the

hypothalamus, could suggest that the hippocampus possesses a critical period that is later than the hypothalamus. This is a reasonable hypothesis considering the hippocampus finishes developing at a later age than the hypothalamus (Bayer, 1980; Jacobson and Gorski, 1981a; Seress, 1985). Alternatively, it may be that the transient expression of ERs in the hippocampus is not related to sexual differentiation at all, but rather play a different role in the development and synaptogenesis of hippocampal neurons.

After describing the distribution of ERα in the developing hippocampus, we asked whether the second form of estrogen receptor, ERβ, was present in the hippocampus and played a role in the development of this brain region as well. ERβ possesses all the properties of a physiological relevant estrogen receptor (Kuiper et al., 1996; Mosselman et al., 1996a; Kuiper et al., 1997) and has been shown to be highly expressed in prostate and ovary with lower expression in brain, uterus, and testis (Mosselman et al., 1996b; Kuiper et al., 1997; Shughrue and Merchenthaler, 2001). The potential role of these different ER subtypes in developmental neurobiology has been established by studies providing clear evidence that, in a given target cell, ERα and β play distinct biological roles (Patrone et al., 2000) suggesting that estrogen can activate selected intracellular signaling pathways depending on the receptor subtype bound. The possibility that ERβ played a role in the developing hippocampus was supported from recent studies using mice lacking ERα and suggested that ERβ may mediate the effects of steroid hormones in a spatial learning task (Cunningham et al., 1998). Additionally, unlike ERα, ERβ mRNA has been shown to be variably spliced resulting in correctly

processed and exon-skipped transcripts that disrupt the normal domain structure of the receptor (Petersen et al., 1998; Inoue et al., 2000; Price et al., 2000). Interestingly, an mRNA splice variant missing exon four has been described in the hippocampus, and cortex, but is absent in nearly all other brain regions examined, including the paraventricular and supraoptic nuclei of the hypothalamus (Price et al., 2000). Thus, the possibility arises that within the developing hippocampus particular ERβ mRNA splice variants are expressed at sufficient levels to significantly influence estrogen mediated transcriptional activation. For example, in transient transfection studies, these splice variants have been shown to be transcribed into functional proteins that have an altered subnuclear localization and transcriptional activities (Price et al., 2001). However, whether ERβ mRNA splice variants exist *in vivo* or not is still unclear, as the tools to analyze the individual variants currently do not exist. Nevertheless, it is enticing to consider the possibility that various ERβ mRNA splice variants could act alone or with the wildtype ERα and ERβ forms resulting in a very specific way by which estrogen influences gene transcription in a particular cell.

Another interesting possibility involves the role of nontraditional ligands that may have ER subtype-selective activity. For instance, the ERβ2 mRNA splice variant has an in-frame insertion of 18 amino acids that disrupts the normal estrogen binding pocket of the receptor. As predicted, this particular ERβ isoform binds 17-β estradiol, the classical ER agonist, with much lower affinity than the wildtype receptor (Petersen et al., 1998). Moreover, a variety of different ligands have been identified to date, which show altered

activity at the various receptor types (Sun et al., 1999). It is possible that during development in the hippocampus, when ERβ2 isoforms are highly expressed, specific ligands may act to create a unique pattern of gene expression required for differentiating neurons. The development of novel ligands for the nuclear receptors has extended beyond neuroendocrinology and has yielded important therapeutic treatments, among them the use of tamoxifen for endocrine therapy of breast cancer (Katzenellenbogen et al., 1995). Curiously, in certain tissues, and after long-term treatment in patients with breast cancer, tamoxifen exhibits unexplained partial agonist activity. Various agents that raise intracellular cAMP levels or stimulate the ras/MAP kinase pathway can similarly cause estrogen receptor activation in the presence of tamoxifen or the absence of any activating ligand (El-Tanani and Green, 1997). It is possible that these molecules, classically considered antagonists can regulate the association with coregulatory molecules with nuclear receptors, including the nature of the ligand, the levels of available coregulator molecules, and the action of diverse protein kinase signaling cascades that modulate the switch from transcriptional repression to activation. In support of the idea that antagonists can influence the action of ERs, we have shown in primary hippocampal cells, ERβ, which is often localized to the cytoplasm, can be translocated to the nucleus following tamoxifen treatment (Fig. 34). However, this effect does not result from estradiol treatment. In chapter four we demonstrated that several ERβ splice variants exist in the developing hippocampus, including a variant missing the

fourth exon. Based on the deletion in this splice variant, it is likely that it is unable to translocated to the nucleus due to a

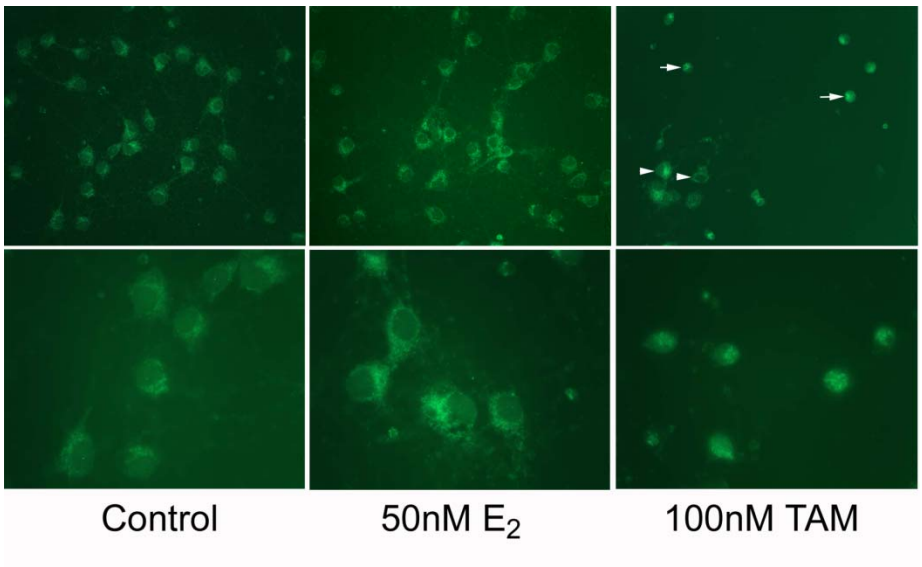


*Figure 34*

disrupted nuclear localization signal. The possibility that tamoxifen can initiate translocation of ERβ raises a variety of questions concerning the role of estrogen receptors in the developing hippocampus.

## *The Role of Brain-Derived Neurotrophic Factor*

More than twenty five years ago, Dominique Toran-Allerand (1976) found that estrogen enhances the growth and arborization of axons and dendrites in hypothalamic

neurons grown in organotypic cultures. In the following years, these initial finding were extended, and growth-promoting effects of estrogen were described in the hippocampus (Gould et al., 1990), midbrain (Reisert et al., 1987), cortex (Garcia-Segura et al., 1989), pituitary (Chun et al., 1998) and spinal cord (VanderHorst and Holstege, 1997). Thus it appears that estrogen has the ability to regulate a wide variety of differentiation processes in neurons that are necessary for the proper development of many different brain structures and connections. However, the mechanisms by which estrogen influences the differentiation of neurons is not entirely clear. One possible mechanism is through an interaction with the neurotrophins. A number of recent studies have demonstrated that neurotrophins play important roles in early development as well as in the later activity-dependent processes important for the final shaping of neural connections (see Barde, 1989; Davies, 1994). Thus, the neurotrophins may play a role in linking functional modifications of synapses to the morphological effects of synaptic stabilization and rearrangement, as observed in the hippocampus. In view of its importance and broad range of activities in neuron populations, brain-derived neurotrophic factor, a member of the neurotrophin family, has gained attention as a potential therapeutic agent for neurological diseases (Lindsay, 1994). Interestingly, estrogen is also an important regulator for BDNF expression in adult rat brain. For example, the level of BDNF mRNA in the hippocampal formation fluctuates across the estrous cycle (Gibbs, 1998) and increases in response to acute estrogen replacement (Singh et al., 1995). It was with these early studies in mind that we chose to examine the effects of estrogen treatment on BDNF gene expression during hippocampal development in the rat.

Over the course of the hormone manipulation studies included in this dissertation, we observed a very curious result concerning the effects of estrogen on BDNF gene expression. This finding is presented in chapter five and demonstrates that following hormone removal resulting from neonatal castration of males, the levels of BDNF mRNA are significantly reduced or even developmentally delayed. Interestingly, in what seemed to be a paradox, estrogen induced decreases in mRNA expression were accompanied by an increase in protein levels. A single injection of estrogen given at the time of castration induces a reversal of this effect such that the levels are returned to those observed in intact animals. The fact that hormone replacement produced an increase in BDNF mRNA and a decrease in BDNF protein in the hippocampus, suggests either an uncoupling of BDNF transcriptional and translational mechanisms, an increase in BDNF protein degradation, or an increase in BDNF release which is then either degraded or transported away, resulting in lower levels of BDNF in the hippocampus. The most likely explanation for the differential effects on BDNF mRNA and protein expression involves evidence demonstrating that BDNF can be transported anterogradely in axons and is released from nerve terminals in a similar fashion to neurotransmitters. However, it is unlikely that the BDNF transport is an estrogen mediated event, as we did not observed any changes in the

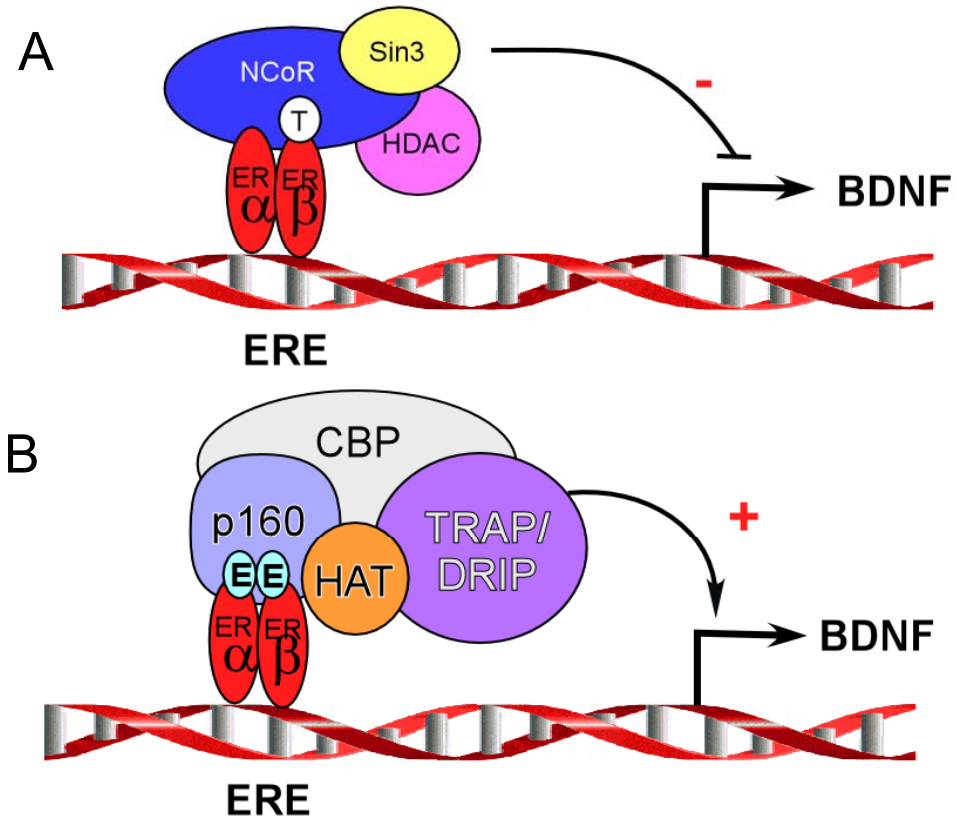


Figure 35: Schematic diagram demonstration the activation of BDNF through a classical estrogen response element via wildtype ERα and/or β. This diagram represents ERα and/or β in an inactivated state [unliganded or in the presence of an antagonist such as tamoxifen (T)] where the corepressor NCoR with its associated HDAC activity acts to silence BDNF transcriptional activation (panel A). Following estrogen (E) binding the ER undergoes a conformational change which releases NCoR and allows binding of p160 coactivators (B). Coactivator binding subsequently recruits additional coregulatory proteins such as CBP with HAT activity. This complex finally recruits the TRAP/DRIP complex which promotes transcriptional activation of BDNF. levels of mRNA or protein

for the BDNF receptor, trkB, over development or following hormone manipulation. With these possibilities in mind, it is then possible to consider that BDNF may even regulate its own production by binding to receptors on pyramidal cells resulting in negative feedback and down regulation of the ligand.

The data presented in this dissertation demonstrate that estrogen significantly affects the levels of BDNF mRNA and protein in the developing hippocampus, and these effects are brain region specific. Given the mounting evidence for effects of BDNF on neuronal connectivity and activity-dependent synaptic plasticity in the brain, it is reasonable to hypothesize that the hormonal effects on BDNF gene expression can contribute to changes in brain structure and function. These influences may, in turn, contribute to the effects of estrogen replacement on cognitive processes, as well as to the ability of estrogen treatment to reduce the risk and severity of Alzheimer's Disease related cognitive decline that have been reported in adult humans.

### *Nuclear Receptor Coregulators*

In recent years, molecular genetics and biochemistry have identified many intermediary components, co-activators, and co-repressors, involved in both ligand-dependent and ligand-independent transcriptional activation following interaction with nuclear receptors (McKenna et al., 1999a; Glass and Rosenfeld, 2000). Recent data show that nuclear receptors, including the estrogen receptors, enhance or inhibit transcription by recruiting an array of these coregulatory proteins to the transcription complex (see Horwitz et al., 1996). Moreover, as the number of putative coactivators and/or

corepressors grows and as functional activities are identified (e.g. histone acetyltransferase or methylation activity), the interplay among the NRs, coactivators and/or corepressors, integrators, and the basal transcriptional apparatus will facilitate, at least in part, an understanding of tissue- and developmental specific gene expression mediated by ligand-activated estrogen receptors. Different cell types may have different relative compositions of these coregulators associated with the nuclear receptors (Misiti et al., 1998). Accordingly, we have investigated the developmental expression of the p160 coactivators, including steroid receptor coactivators (SRC) 1-3, in the rat brain. Interestingly, the expression pattern for the p160 coactivators in the developing brain is highly variable, suggesting that while the coactivators are widely expressed, their relative expression is dependent upon the brain region and ontogenetic period examined. In this dissertation, we have demonstrated that estrogen receptors and steroid receptor coactivators are each expressed in the developing hippocampus. Indeed, the interaction between these proteins is further supported in figure 36 demonstration that over 81% of primary

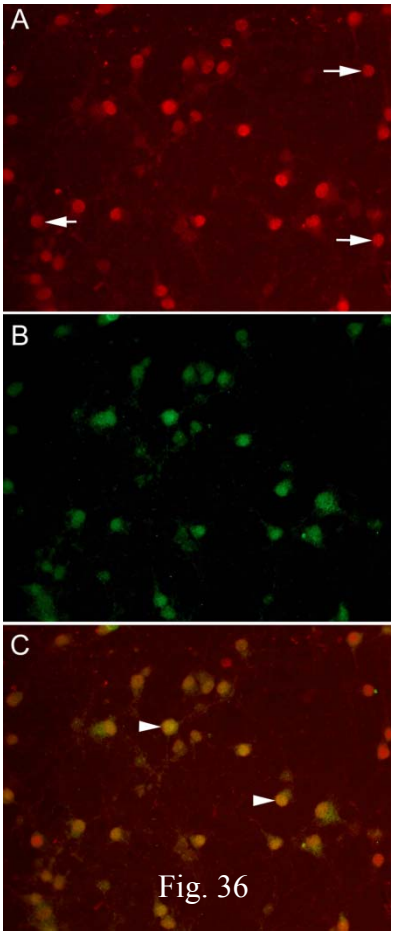

Fig. 36

hippocampal cells containing SRC-1 (red) also contain ERα (green). It appears then, that within the developing brain, a highly complex system for transcriptional regulation exists. This complex includes steroid responsive genes, nuclear receptors, coregulatory proteins and the general transcriptional machinery.

How estrogen receptors interface with the p160 coactivator system, for example, is an intriguing question, but given that nuclear receptors do not contain histone acetyltransferase activity, one could envision a two-step process. In this hypothetical model, chromatin remodeling coactivators such as CBP and p300, either bound directly to steroid receptors or through ligand-recruited coactivators such as the p160 coactivators could open up the chromatin to then allow the nuclear receptors to act directly on the preinitiation complex. Thus, the specific recruitment of a complex with histone acetyltransferase activity to a promotor may play a critical role in overcoming repressive effects of chromatin structure on transcription (Struhl, 1998). It is even more intriguing to speculate that coactivator proteins could interact in a ligand independent fashion with the AF-2 domain of the estrogen receptor to influence transcriptional activation. For instance, concerning the BDNF gene, it is convenient to think that following ligand binding and recruitment of specific coactivator proteins, the wildtype estrogen receptors bind to the estrogen response element in the BDNF promotor to initiate transcription (Fig 35). An alternative, albeit much more complex, scenario involves the ligand independent interaction of an ERβ splice variant, likely ERβ2 or ERβ2δ3, following the phosphorylation of the AF-1 domain via activation of the AKT or ERK pathway.

Following this phosphorylation event an array of coactivator proteins including the p160s and CBP could be recruited the estrogen receptor. Finally, the CBP

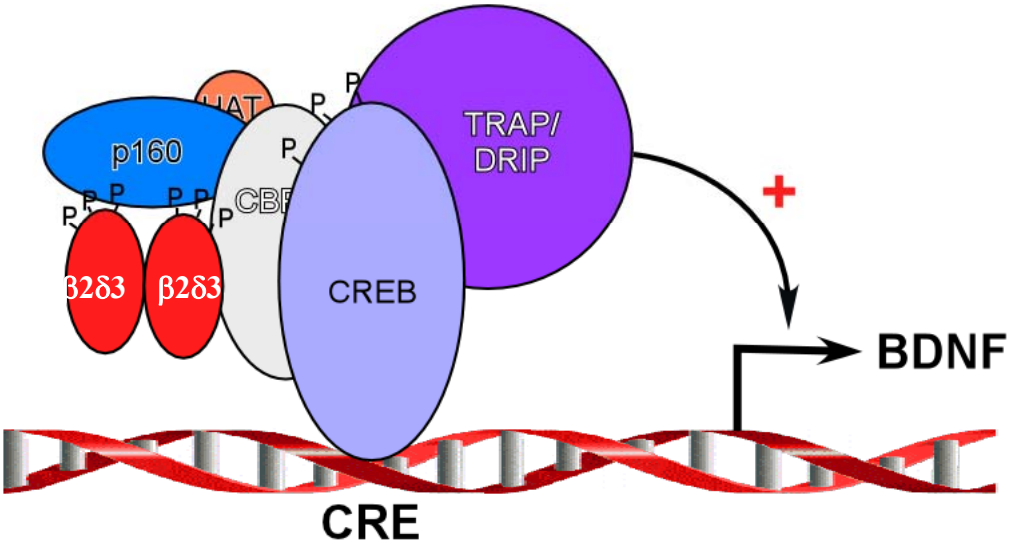


Figure 37: A schematic diagram showing the activation of BDNF through a non-classical pathway involving nuclear receptor coactivators. This hypothetical diagram represents a scenario in which ERβ splice variants could activate BDNF transcription in an agonist independent (or antagonist stimulated) fashion. Following phosphorylation of ER activation domains, steroid receptor coactivators can interact with the receptor. Through a subsequent series of protein-protein interactions, additional coregulatory proteins could be recruited resulting ultimately in gene activation. In this particular example, CREB

(cAMP response element) is the requisite transcription factor for transcriptional activation.

bound complex could interact with the CBP responsive element in the BDNF promotor to initiate transcription (Fig 37). However, concerning this alternative scenario, it should be noted that no evidence of ligand independent activation of ERβ2 splice variants has been reported, making the first hypothetical situation even more attractive.

A further understanding of which intermediary factors are involved in the transcriptional control of particular target genes, including BDNF, will require a detailed analysis of genes in a chromatin complex within cells. These studies will need to be conducted in concert with the continued development of transcription systems that respond to multiple activators in a chromatin context *in vitro*. When combined, these types of experiments will allow for further identification of novel cofactors involved in gene transcription. They will also assist in the dissection and elucidation of the direct biochemical functions of particular chromatin modifying, remodeling, or general cofactors in coordinating the action of nuclear receptors and other classes of activators and repressors at complex promoters. In addition to potential enhancer-induced cooperativity, such experiments may also reveal novel activator specific or gene-selective mechanisms of transcriptional control in response to multiple stimuli and, taken together, should increase our understanding of cellular proliferation and differentiation.

## *Conclusion*

Brain development involves a precise program of gene activation that establish specific neuronal phenotypes and the intricate patterns of connections between them. Understanding the mechanisms underlying these selective programs of gene activation and repression that define the maturation of neurons represent a fundamental question in neurobiology. However, despite considerable evidence that gonadal hormones influence the function and organization of circuits in the brain, the molecular signals that regulate the structural and functional changes in connections during development remain largely obscure. Estrogen affects the nervous system in ways that extend beyond its essential actions of regulating gonadotropins and prolactin secretion and modulating sexual behavior. The diversity of these effects implies an involvement of brain regions outside of the hypothalamus, a traditional site for the study of estrogen receptors and their role in the control of reproductive function. Such extrahypothalamic sites include, for example, the hippocampus, basal forebrain and cerebral cortex. These non-hypothalamic brain regions are also particularly susceptible to damage resulting from stroke or neurodegenerative disease. Moreover, in humans, epidemiological studies and clinical experiments have suggested that estrogen replacement therapy reduces the risk for neurodegenerative diseases that target hippocampal neurons, such as Alzheimer's disease (Henderson et al., 1996; Asthana et al., 2001). Additional studies have shown that estrogen treatment has positive outcomes on cognition and memory (see Sherwin, 1994; Fink et al., 1996; Sherwin, 1997). Both of these effects are possibly mediated through interactions with the neurotrophins. Thus, the implications of the studies presented in

this dissertation extend beyond endocrine neurobiology alone. They demonstrate that estrogen not only plays an important role in the hippocampus, but that the influences of this steroid hormone are mediated during development at a critical time when hippocampal neurons are differentiating and undergoing synaptogenesis.

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# VITA

The author, Derek Tyler Solum, was born on February 7, 1972, in Salt Lake City, Utah to Dallas and Marcia Solum. He completed his secondary education at Murray High School, Murray, Utah, in 1990. In fall, 1990, Derek entered the University of Utah where he was the recipient of a Howard Hughes undergraduate research scholarship. As an undergraduate, Derek worked in the laboratory of Dr. Thomas N. Parks in the Department of Anatomy and Neurobiology where he studied the development of the avian auditory system. Derek graduated from the University of Utah in 1995 with an honors Bachelor of Science degree in Biology.

In August 1996, Derek entered the doctorate program in the Department of Cell Biology, Neurobiology and Anatomy at Loyola University Chicago. In 1997, he joined the laboratory of Dr. Robert J. Handa to pursue research in developmental neuroendocrinology. He was the recipient of a Loyola University Basic Science Fellowship from 1996-1999. In 1999, Derek successfully competed for a pre-doctoral National Research Services Award from the Public Health Service of the National Institutes of Health. Derek is a member of the Society for Neuroscience, the Endocrine Society, and the American Neuroendocrinology Society.

Derek has accepted a postdoctoral position in the laboratory of Dr. Michael G. Rosenfeld in the Department of Molecular Medicine at the University of California, San

Diego. There he will pursue research in the molecular mechanisms of DNA transcription.